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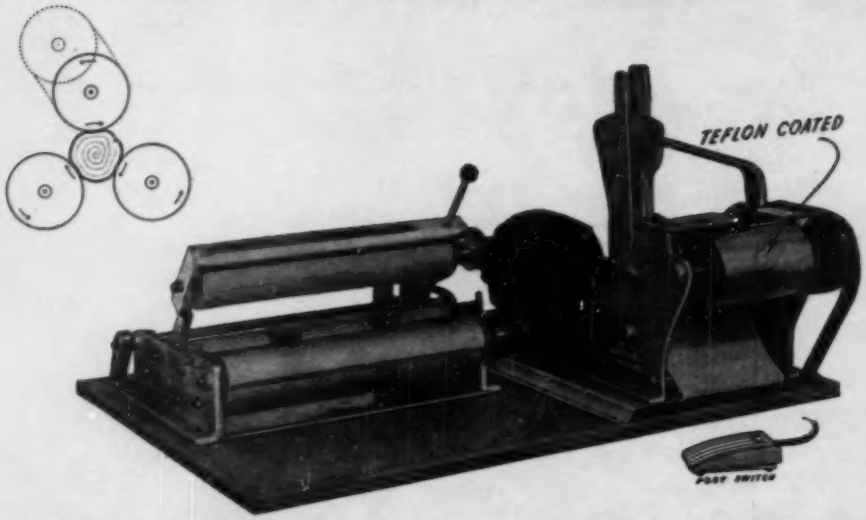

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EFFECT OF FLOUR LIPIDS AND SOME LIPID DERIVATIVES ON COOKIE-BAKING CHARACTERISTICS OF LIPID-FREE FLOURS[1]

E. W. COLE, D. K. MECHAM, AND J. W. PENCE[2]

ABSTRACT

Cookies baked from flours which had been extracted with water-saturated *n*-butyl alcohol were generally darker brown in color and had diameters appreciably smaller than those baked from untreated flours. The restoration of lipids to the extracted flours resulted in an increase in cookie diameter and an apparently complete recovery of the characteristics of cookies baked from the original flours. Approximately three-fourths of the total lipid must be removed from a flour before definite changes in cookie-baking behavior (top grain and color) are noted. When the lipids of two flours (which differed in cookie-spread properties) were interchanged, the diameter of the cookies was the same as that of cookies from untreated flours; that is, the diameter appears to depend on constituents other than flour lipids. When lipid-free flours were supplemented with the phosphorus-free fraction from flour oil, no improvement occurred in the cookie-baking quality of these flours; the phosphorus-containing fraction, however, produced complete recovery of cookie characteristics. Soybean or corn phosphatide preparations and a purified soybean lecithin also brought about a similar improvement.

Investigations concerned with the relationship of wheat flour lipids to the baking quality of flours have been carried on by several workers, among whom Sullivan *et al.* (13,14) and Cookson *et al.* (3,4) may be mentioned. Their work, however, has been confined chiefly to a study of the role of flour lipids in the breadmaking behavior of hard wheat

[1] Manuscript received May 11, 1959. Presented at the 44th annual meeting, Washington, D. C., May 1959.

[2] Western Regional Research Laboratory, Albany, California; laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

flours, and little attention has been given to soft wheat flour varieties, which are the types best suited for pastries and cookies. The present study was undertaken to determine the extent to which cookie-baking properties of soft wheat flours are affected by flour lipids.

One difficulty encountered in the evaluation of flour lipids was that of the preparation of suitable flour samples which were free of lipids. In general, those procedures used to remove all of the lipids from flours also produced deleterious effects on gluten and other constituents of flour. Mecham and Mohammad (8) obtained an essentially complete removal of lipids from flour through use of water-saturated *n*-butyl alcohol as the extracting solvent. The few observations which these workers made on bread-baking behavior of flours indicated that extraction with this solvent resulted in a pronounced loss of loaf volume, but as much as two-thirds of the loss could be regained by replacement of the extracted material. These encouraging results prompted the use of water-saturated *n*-butyl alcohol as the extraction solvent for preparation of lipid-free flour samples used in the present study. Such solvents as carbon tetrachloride, petroleum ether, and chloroform were incapable of extracting all of the lipid from the flour. They were used in this series of experiments, however, to provide additional comparative information. The results of the cookie-baking tests which were made in this study and the effect of various lipids on the cookie-baking characteristics of lipid-free flours are described in this paper.

Materials and Methods

One hard and two soft wheat flours, all of which were commercially milled, and one hard wheat flour, which was experimentally milled from a pure variety (Rio), were used in this work. All were unbleached.

Lipid Extraction Procedures. Lipids were extracted from the flours with water-saturated *n*-butyl alcohol by the procedure of Mecham and Mohammad (8) with some modifications. A 500-g. sample of flour was placed in a beaker and covered with 1200 ml. of water-saturated *n*-butyl alcohol. This mixture was stirred to disperse all of the flour and allowed to stand for approximately 30 minutes. Then the clear supernatant was decanted and filtered through S&S No. 588 filter paper. The flour residue was extracted five more times in the manner just described, with 400-ml. portions of the solvent each time. Finally, the residue was placed on the filter paper and allowed to drain free of excess solvent. The final filtrate was tested for esters qualitatively with the hydroxamic acid method of Hestrin (6). Two milliliters of the filtrate from the extracted flour were added to 3 ml. of methanol. The hydroxamic acid test was made on this solution. The extraction

of lipid was presumed complete when a negative hydroxamic acid test for fatty acid esters was obtained on the filtrate.

To remove residual *n*-butyl alcohol, the extracted flour was dried *in vacuo* at 40°C. for approximately 16 hours. The vacuum-dried material was humidified in a proofing cabinet to a moisture content of approximately 20% and again dried *in vacuo* to approximately 2% moisture. Finally, the dried sample was ground in a small Wiley Mill[3] to pass the No. 20 mesh screen. Lipid-free samples prepared in this way had no detectable odor of butyl alcohol when they were moistened or mixed into a cookie dough. The flour lipid was recovered from extracts by removing the butyl alcohol with a rotary evaporator at a temperature not exceeding 40°C.

When carbon tetrachloride or petroleum ether was used to extract flour lipids, 500 g. of the flour were extracted by percolation in a glass column, using about 1500 ml. of solvent.

Reconstitution Procedures. To reconstitute the flours, desired proportions of lipid were dispersed in water-saturated *n*-butyl alcohol and added to the extracted sample. The residual solvent was removed by drying as described previously.

To reconstitute the flours which had been extracted with carbon tetrachloride or petroleum ether, the lipid was dispersed in the original extracting solvent and added to the extracted flour sample. This reconstituted flour was then spread on trays, and residual solvent was allowed to evaporate at room temperature. All flour samples were humidified to a moisture content of about 12% before they were mixed into doughs.

Fractionation of Flour Lipids. The lipid material obtained from a commercially milled flour by extraction with water-saturated *n*-butyl alcohol was freed of all solvent and dried in a desiccator *in vacuo* over phosphorus pentoxide prior to fractionation. The dried lipid was then dispersed in petroleum ether (b.p. 30°–60°C.). An insoluble fraction precipitated as a light-tan powder and was removed by centrifugation. The lipid soluble in petroleum ether was freed of solvent by evaporation and dissolved in chloroform. The chloroform solution of the lipid was then separated on a silicic acid:celite column into two fractions, one containing phosphorus and the other phosphorus-free, according to the procedure of Borgstrom (2). The fraction free of phosphorus was obtained by passing chloroform through the column containing the absorbed lipid until no more lipid material was found in the eluate. The phosphorus-containing fraction remaining on the

[3] Mention of trade names or products does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

column was then eluted wth methanol.

The fraction which contained phosphorus was further separated into various components with the gradient elution technique described by Weiss (15). The glass column used in this procedure was packed with 120 g. of the silicic acid:celite absorbant having an absorption capacity of 0.6 mg. phosphorus per gram of absorbant. The mixing chamber, attached above the column and equipped with a magnetic stirrer, contained 850 ml. of chloroform. Methanol was added to the chamber dropwise under a nitrogen pressure of about 3 p.s.i. The rate of flow of solvent eluate from the column was approximately one drop per 8 seconds. The eluate was collected as 5-ml. portions in test tubes. The tubes were grouped in successive units of four and the contents of the four combined as one fraction for analysis.

The amounts of solids in the different fractions were obtained by weight.

Analytical Methods. Total phosphorus was determined by the method of Allen (1). Nitrogen was determined by a standard micro-Kjeldahl method. Sugars were quantitatively determined by the anthrone method of Radin *et al.* (12) using glucose as the standard; and qualitatively determined using the paper chromatographic procedure of Williams *et al.* (16).

Cookies were baked by the micro-baking procedure No. III of Finney *et al.* (5) using 40 g. of flour per bake. Cookie quality was expressed as the average diameter and diameter-to-thickness ratio. The reflectance of the cookies was determined using a Hunter Color and Color Difference Meter with standard filter No. SKC-SBC-31.

Results and Discussion

Characteristics of Cookies Baked from Lipid-Free Flours. Cookies baked from flours whose lipids had been extracted with water-saturated *n*-butyl alcohol had consistently smaller diameters, exhibited more compact, hard-appearing top surfaces, and were darker in color than the cookies from the control flour. Restoration of lipids to the extracted flours resulted in an increase of the cookie diameter and a regain of the characteristics of cookies baked from the control. Figure 1 shows the cookies baked from a soft wheat commercial flour whose lipids were extracted with petroleum ether, carbon tetrachloride, and water-saturated *n*-butyl alcohol. The diameters and the diameter-to-thickness ratios of these cookies are shown in Table I. Carbon tetrachloride and petroleum ether extraction produced only minor changes in cookie-baking quality of the flour when compared with the effect produced by water-saturated *n*-butyl alcohol extraction. The amounts

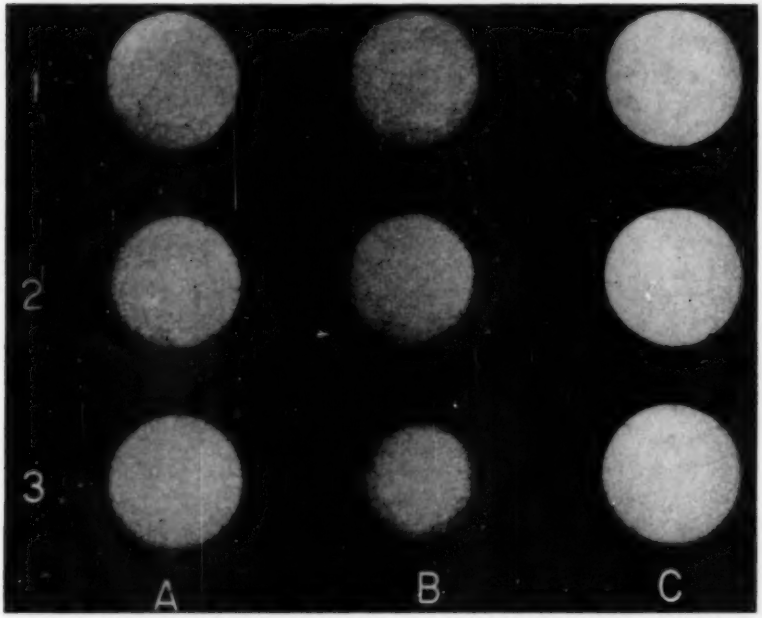

Fig. 1. Cookies baked from a soft wheat commercial flour extracted with different solvents. Top row: A, untreated; B, extracted with petroleum ether; C, reconstituted with petroleum ether extract. Middle row: A, untreated; B, extracted with carbon tetrachloride; C, reconstituted with carbon tetrachloride extract. Bottom row: A, untreated; B, extracted with water-saturated *n*-butyl alcohol; C, reconstituted with water-saturated *n*-butyl alcohol extract.

TABLE I

EFFECT OF SOLVENT EXTRACTION ON COOKIE SPREAD OF A SOFT WHEAT COMMERCIAL FLOUR

FLOUR SAMPLE	COOKIE DIAMETER[a]	DIAMETER/THICKNESS
	cm	cm
Untreated	8.79	12.0
Extracted with petroleum ether	8.49	11.0
Extracted with petroleum ether and reconstituted	8.61	12.0
Extracted with carbon tetrachloride	8.41	—
Extracted with carbon tetrachloride and reconstituted	8.52	12.0
Extracted with water-saturated *n*-butyl alcohol	7.49	6.8
Extracted with water-saturated *n*-butyl alcohol and reconstituted	8.94	13.6

[a] Average diameter in cm. of at least four cookies from two baking experiments.

of lipid removed from this flour (as-is basis) by these three solvents were as follows: petroleum ether, 0.88%; carbon tetrachloride, 0.97%; and water-saturated *n*-butyl alcohol, 2.0%.

Similar changes occurred in the cookie-baking performance of hard wheat flours as well as the soft wheat flours examined when their lipids were removed with water-saturated *n*-butyl alcohol:

Flour sample	*Cookie Diameter*	*Diameter/Thickness*
	cm	
Commercial spring wheat flour		
Untreated	8.01	8.9
Extracted	7.46	7.4
Reconstituted	8.32	10.5

The changes, however, were more pronounced with the soft wheat flours. When lipids were removed from both types of flours, the decrease was greatest in the spread of cookies baked from the soft wheat flour.

Effect of Lipid Interchange between Flours. Although the soft wheat commercial flour and the Rio variety hard wheat flour contain approximately the same quantity of protein, they produced cookies with different diameters. Cookies were baked from lipid-free portions of these two flours after reconstitution in which their total lipids were interchanged. The diameters obtained for these cookies were as follows:

Flour sample	*Cookie Diameter*
	cm
Commercial soft wheat flour (6.7% protein)	8.79
Extracted	7.53
Reconstituted with own lipids	8.85
Reconstituted with Rio lipids	8.90
Rio variety (6.9% protein)	8.23
Extracted	7.46
Reconstituted with own lipids	8.54
Reconstituted with commercial soft wheat flour lipids	8.52

According to these results, when the flour lipids were interchanged, the average diameter of the cookies from the flour containing interchanged lipid was the same as the diameter observed in cookies from the reconstituted flour. Since the soft wheat flour used in these experiments was not from a pure-variety lot of wheat, it is not possible to determine whether flour lipids affect cookie spread strictly from a varietal standpoint. The results above, however, suggest that properties of flour lipids from soft and hard wheat flours are similar in improving the behavior of extracted flours.

Effect of Flour Lipid Concentration on Extracted Flours. The amount of flour lipid which was added to lipid-free flour in order to produce a cookie with top grain and color closely resembling the

control cookie was found to be less than the total amount of lipid originally extracted from the flour. Flour lipid in amounts varying from 0 to 100% of the total extractable lipid was added back to portions of the lipid-free commercial soft wheat flour. Table II shows the effect produced on the color and diameters of cookies from the

TABLE II

EFFECT OF FLOUR LIPID CONCENTRATION ON CHARACTERISTICS OF COOKIES BAKED FROM A SOFT WHEAT COMMERCIAL FLOUR EXTRACTED WITH WATER-SATURATED *n*-BUTYL ALCOHOL

TOTAL FLOUR LIPIDS ADDED TO EXTRACTED FLOUR	COOKIE DIAMETER[a]	REFLECTANCE OF COOKIES
%	*cm*	%
100	8.52	47.4
50	8.24	46.5
20	7.99	47.6
5	8.02	42.9
1	7.82	35.7
0	7.46	37.8

[a] Average diameter in cm. of at least four cookies from two baking experiments. The average diameter of cookies baked from the untreated flour was 8.50 cm., and the reflectance was 47.2%.

series in which the lipid concentration was varied. Cookies containing less than one-fifth of the total lipids showed a distinct difference in color and loss of top grain, whereas cookies with higher lipid concentrations closely resembled the control. In general, with each decrease in lipid concentration, there was a decrease in cookie diameter. When a similar concentration study was made with the commercial hard wheat flour of 13% protein, the amount of lipid needed to restore the optimum characteristics (top grain and color) to the cookie was found to be approximately one-fourth of the total extractable lipid. Since about three-fourths of the total lipid had to be removed from a flour before definite changes in the cookie-baking behavior were noted, a possible explanation is furnished as to why extraction of flours with carbon tetrachloride or petroleum ether has no effect on cookie-baking quality of flours. As was indicated previously, these solvents remove only about one-half of the total extractable lipid from flours. Moreover, only small fractions of the total extractable lipid phosphorus are removed by these solvents.

As was indicated earlier in this paper, the cookies baked from lipid-free flours were consistently darker in color than the control. Table II shows that cookies containing less than one-fifth of the total extractable lipids exhibited a corresponding increase in brown color compared with the control. Cookies from extracted flours with flour

lipids or phosphatides added back in normal amounts possessed the original light-yellow color of the control. These lipids thus seem to interfere somehow in the browning reactions that normally occur during baking.

Effect of Flour Lipid Fractions on Baking Quality of Extracted Flours. All of the lipid fractions obtained from the commercially milled flour are listed in Table III along with the analytical data for sugar, nitrogen, and phosphorus content.

TABLE III

ANALYSIS OF FLOUR LIPID FRACTIONS

FRACTION	APPROXIMATE TOTAL LIPID	NITROGEN	PHOSPHORUS	APPROXIMATE GLUCOSE	NITROGEN/PHOSPHORUS RATIO
	%	%	%	%	
Petroleum ether, insoluble fraction	9.6	1.43	0.11	80	33.0
Petroleum ether, soluble fraction	90.5	—	0.75	18	
Tube group 7–12	34.0	0.46	0.69	23	1.5
Tube group 13–16	3.8	3.8	1.18	92	7.1
Tube group 17–20	3.6	1.4	0.23	64	13.5
Phosphorus-free fraction	49.0	0.0	0.0	0	...

The fraction which precipitated as the result of dispersing the total flour lipid in petroleum ether was light tan in color, contained chiefly sugars, and was partially soluble in water. Acid hydrolysis of this solid followed by paper chromatographic analysis for sugars only showed the presence of glucose and fructose. When added to lipid-free flours, this fraction produced no improvement in the cookie spread (Table IV). The first fraction eluted from the silicic acid:celite column with

TABLE IV

EFFECT OF FLOUR LIPID FRACTIONS ON DIAMETER OF COOKIES BAKED FROM LIPID-FREE FLOURS

LIPID FRACTION[a]	COOKIE DIAMETER[b]
	cm
Petroleum ether, insoluble fraction	7.31
Phosphorus-free fraction	7.74
Tube groups 7–12	8.33
Tube groups 13–16	7.41
Tube groups 17–20	7.51

[a] Lipid fractions were added to flour extracted with water-saturated *n*-butyl alcohol in the same proportionate amounts as were isolated from total flour lipid.

[b] Average diameter in cm. of at least four cookies from two baking experiments, each with 40 g. of extracted flour supplemented with the above lipid fractions.

chloroform contained no phosphorus, sugars, or nitrogen. This fraction probably contained mostly neutral fats and esters and did not appreciably improve the quality and spread of cookies baked from lipid-free flours to which the fraction had been added back. These cookies had a greasy appearance and were dark brown in color.

The second fraction eluted from the column with methanol was further separated by gradient elution into one major and two minor subfractions. Figure 2 shows the distribution curve for the solids eluted from the column. The contents of the fractions in tubes 7–12 inclusive, 13–16 inclusive, and 17–20 inclusive (Fig. 2 and Table III) were

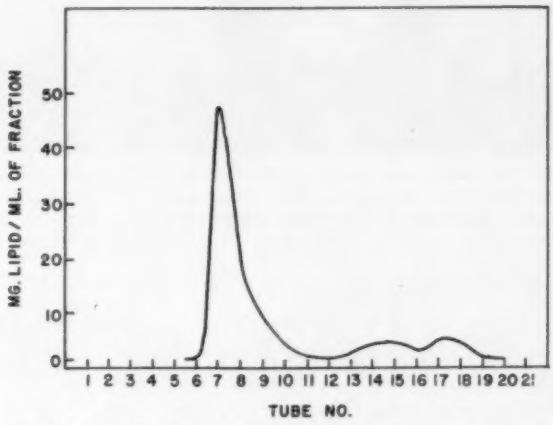

Fig. 2. A weight distribution curve of the phosphorus-containing fractions of flour lipids obtained by elution with chloroform-methanol.

combined into three groups: A, B, and C, respectively. Large values were found for the nitrogen-to-phosphorus ratios of the solids in groups B and C, which suggested that these groups were contaminated with nonphospholipid nitrogen. One group (A), however, which contained phosphorus to the extent of one-third of the phosphorus in the total lipid, had a nitrogen-phosphorus ratio of 1.5 and may have been enriched with phospholipids, such as lecithin or cephalin (a ratio of 1.0 indicated presence of phospholipid only). Group A contained the constituents responsible for cookie spread and quality as shown in Table IV and Fig. 3. The remaining two groups produced no appreciable improvement. Group C on standing at 4°C. for several days deposited a white crystalline material. Hydrolysis of the material followed by paper-chromatographic sugar analysis showed presence of glucose and fructose. No further attempts were made to determine the

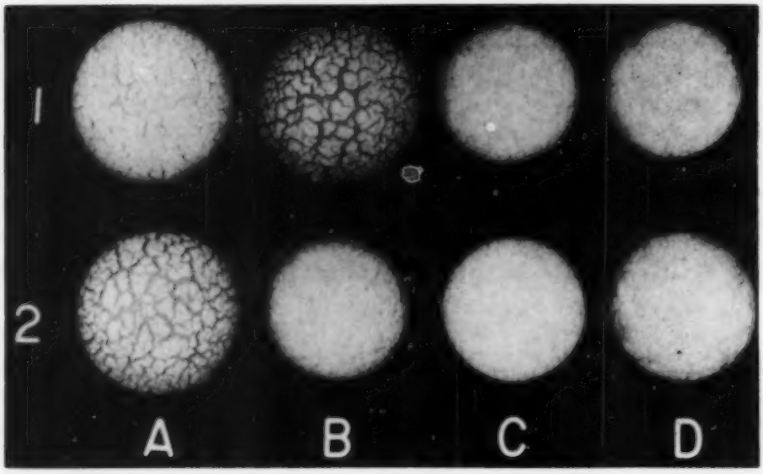

Fig. 3. Effect of flour lipids on the cookie-baking properties of a flour extracted with water-saturated *n*-butyl alcohol. Fractions added: 1A (untreated flour); 1B, total flour lipid; 1C, none; 1D, petroleum ether-insoluble solids; 2A, tube group 7–12; 2B, tube group 13–16; 2C, tube group 17–20; 2D, nonphosphorus fraction.

nature of the constituents in these groups.

Effect of Other Lipids on Baking Quality of Extracted Flours. To determine whether improvement in cookie-baking performance of extracted flours, brought about by flour lipid, was due to a property characteristic only of flour lipid or whether lipids foreign to flour could also bring about cookie-baking improvement, a variety of lipids, both natural and synthetic, were added individually to portions of lipid-free flours. Tables V and VI list some of the lipids used and the

TABLE V

DIAMETERS OF COOKIES BAKED FROM A SOFT WHEAT COMMERCIAL FLOUR EXTRACTED WITH WATER-SATURATED *n*-BUTYL ALCOHOL AND SUPPLEMENTED WITH VARIOUS LIPIDS

LIPID ADDED TO EXTRACTED FLOUR[a]	COOKIE DIAMETER[b]
	cm
(Untreated)	8.46
Total flour lipid	8.53
None	7.42
Sucrose fatty acid ester	8.26
Vegetable tocopherols	7.76
Mono-olein	7.89
Wheat germ oil	8.66

[a] Lipid added to the extent of 2.3% to the lipid-free flour (dry-weight basis).
[b] Average diameter in cm. of at least four cookies from two baking experiments, each with 40 g. of extracted flour supplemented with the above lipids.

TABLE VI

DIAMETERS OF COOKIES BAKED FROM A SOFT WHEAT COMMERCIAL FLOUR EXTRACTED WITH WATER-SATURATED *n*-BUTYL ALCOHOL AND SUPPLEMENTED WITH PHOSPHATIDES

LIPID ADDED TO EXTRACTED FLOUR	AMOUNT ADDED[a]	COOKIE DIAMETER[b]
		cm
(Untreated)		8.20
Total flour lipid	2.30	8.27
None	0.0	7.45
Soybean phosphatide	0.59	7.98
Corn phosphatide	0.75	8.02
Hydroxylated soybean lecithin	0.92	8.28
Alcohol-soluble soybean phosphatides	0.59	8.10
Alcohol-insoluble phosphatides	0.59	8.00
Purified soybean lecithin	0.88	8.15

[a] Percent lipid added to the lipid-free flour (dry wt. basis). The phosphatide preparations were added in quantities containing the same amount of phosphorus as was contained in the natural flour lipid.
[b] Average diameter, in cm. of at least four cookies baked from two baking experiments, each with 40 g. of extracted flour supplemented with the above lipids.

diameters of cookies baked from extracted flours supplemented with these lipids. Such constituents as wheat germ lipids, soy phosphatides, and a sucrose fatty acid ester improved the baking quality of the extracted flours (see Fig. 4 also). An extracted flour to which Tween 80 had been added produced a cookie dough that was sticky and unmanageable, and the baked cookies were very dark in color and irregular in shape.

The results given in Table VI show that phospholipids improved the baking characteristics of the extracted flour, because cookies baked from all doughs containing the crude phosphatide preparations showed an improvement in spread, color, and top grain, as compared with cookies from the extracted flour alone. To add support to the contention that phospholipids exert an improving action, a sample of lecithin prepared from a crude soybean preparation by the procedure of Pangborn (11) and Macpherson (7) was added to the extracted flour. Cookies baked from this flour (Fig. 4) closely resembled the control in all respects.

Whether the flour lipids exert an improving action by themselves or in combination with other constituents of lipid-free flour, such as starch or protein, is not known. There is evidence that a phospholipid-protein complex exists in flour dough, and suggestions have been made that this complex is necessary for optimum bread-baking behavior (9,10). The same type of complex may also play an important part in the cookie-baking behavior of flour.

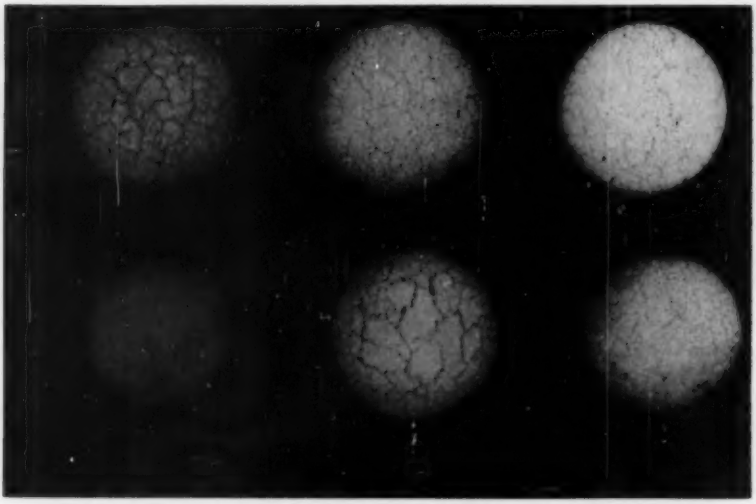

Fig. 4. Effect of various lipids on the cookie-baking properties of a flour extracted with water-saturated *n*-butyl alcohol. Lipids added: 1A, flour lipid; 1B, soybean lecithin, crude; 1C, soybean lecithin, purified; 2A, none; 2B, sucrose fatty acid ester; 2C, vegetable tocopherols.

Acknowledgments

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HEMICELLULOSES OF THE CEMENTING LAYER AND OF SOME CELL WALLS OF THE CORN KERNEL[1]

H. L. Seckinger, M. J. Wolf, and Majel M. MacMasters

ABSTRACT

Hemicellulose fractions of the cementing layer and of various cell walls in the corn kernel were analyzed by quantitative paper chromatography. Dilute acid hydrolysis of the cementing layer showed that it contained 60% polysaccharide composed of 55% arabinose, 31% xylose, and 14% glucose. Total nitrogen analysis indicated a protein content of 30%.

Cell walls of the scutellar parenchyma, scutellar epithelium, and endosperm were found to contain 50, 30, and 20% hemicellulose, respectively. On hydrolysis, hemicellulose of the endosperm cell walls yielded 34% arabinose, 35% xylose, and 31% glucose in contrast to the hemicellulose of the cell walls of scutellar parenchyma and epithelium, each of which yielded about 60% arabinose, 30% xylose, and 5% galactose. In addition, a small amount of glucose was found in the hydrolysate of the cell walls of the scutellar parenchyma. No pectic substances were found in any of the cell walls examined.

The properties of cell walls of corn kernels are of considerable importance to the processor, because cell walls must be broken in either wet-milling for starch production or in dry-milling for the production of grits and meal.

Walls of the epithelial cells of the scutellum, as shown previously (9), are more easily degraded by enzymes than is the cementing layer

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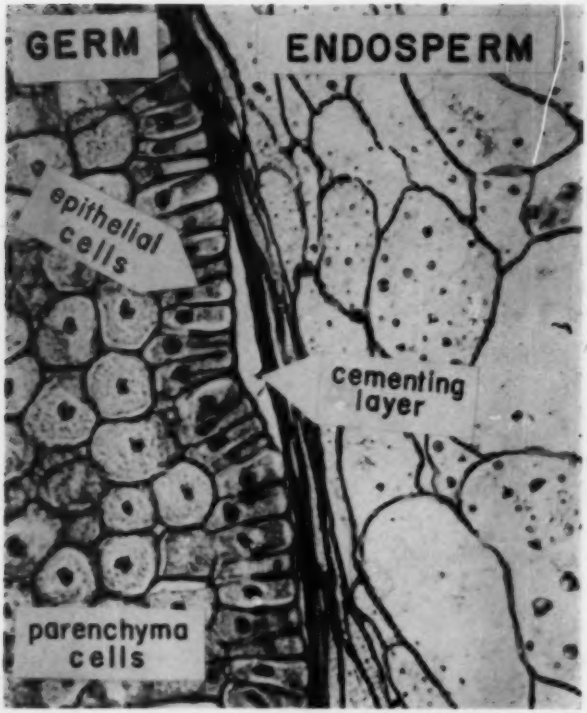

Fig. 1. Cross-section of the corn kernel through the region where the germ and endosperm meet, showing some of the cells which were analyzed. Mag. 250×.

between germ and endosperm. (These structures are illustrated in Fig. 1.) Even partial degradation of the epithelial cell walls helps to loosen the adhesion of the germ to the endosperm and facilitates degermination.

There are a number of well-known histochemical methods by which the chemical constituents of cell walls may be identified. A few of the methods can be used to obtain very rough estimates of the comparative amounts of the various constituents that are present, but histochemical tests are of chief value for qualitative identification of the constituents and determination of their locations *in situ*.

To study the quantitative composition of the cementing layer and adjacent tissues, methods were adapted for analysis of very small amounts of isolated cell walls. Emphasis was placed on the hemicellulose or pentosan fraction, because previous work had shown that this material was more readily degraded than other cell-wall constituents.

It is therefore of primary significance in degermination and other processes which involve cellular degradations. Methods were first tested on cell walls that could be more easily isolated in larger quantity than could material from the cementing layer region. These preliminary studies, which were made on cell walls from the aleurone layer and from the starchy endosperm, not only permitted refinement of the methods, but also gave data of potential value to the processor. In this paper, information thus obtained is combined with results of studies on wall materials in the cementing layer region to provide a more comprehensive view of cell-wall composition throughout the raw material used by the corn milling industries.

Materials and Methods

Isolation of Cementing Layer. Yellow dent corn (U.S. 13 and Ill. 1277 varieties) was steeped in distilled water for 16 hours at room temperature, then 120-μ cross-sections were cut on a freezing microtome. Although the cementing material extends over the whole area between the germ and endosperm, it is too thin in most places to isolate under the dissecting microscope. However, masses of cementing material occur at gland formations or other irregularities of the epithelium (Fig. 1), and it was from these sites that about 200 γ of material were obtained from some 5,000 selected sections.

Isolation of Cell Walls. A network of cell walls was easily obtained by removing the contents from thin sections of tissue. The size and shape of the cells determined how thick and in what direction to cut the sections from the corn kernels. To aid in separation of starch and protein from endosperm cell walls, 30-μ fresh sections were shaken in a 0.5% solution of sodium hypochlorite for a few minutes. Residual starch was gelatinized and solubilized by treatment with a malt alpha-amylase preparation.

Cell walls of the scutellar parenchyma and epithelial cells were obtained from 16-μ sections of germ cut perpendicular to the long axis of the epithelial cells. After the network of cell walls had been defatted in petroleum ether, it was inspected microscopically to confirm that all protein and starch had been removed.

Cross cells were scraped from the inner surface of the pericarp and extracted with hot 50% ethanol.

Aleurone cells were scraped from the outer surface of dehulled kernels and defatted with hot 85% methanol.

The pericarp was hand-dissected, then extracted with hot 0.5% ammonium oxalate to remove pectic substances, starch, and solubles.

Hydrolysis. Hydrolysis was carried out in a small, sealed tube con-

taining 40–110 γ of air-dried cementing material or cell walls and 0.2 ml. of 2% sulfuric acid. The tubes were autoclaved for 5 hours at 120°C., the hydrolysate was neutralized by first adding a saturated solution of barium hydroxide, and the pH was adjusted to 6.2–6.8 with powdered barium carbonate. The mixture was transferred to a strip of filter paper 4 by 15 cm. and eluted for 3 hours (4). This step removed the sugars while leaving the insoluble barium sulfate on the paper. The eluted volume (about 0.5 ml.) was evaporated to a small drop for spotting chromatograms.

Paper Chromatography. Both qualitative and quantitative paper chromatography was conducted by the descending method, with the use of known sugar standards and a solvent system of *n*-butanol, pyridine, water, 6:4:3 (2).

For quantitative studies, a single solution containing three sugars was applied on the chromatogram with a Gilmont[2] micropipet. The solution was spotted along the starting line in 0.5-μl. increments up to 2 μl., which deposited 2.5–10 γ of sugars. Three reference spots were used to determine the concentration curves; a fourth spot contained the entire neutralized hydrolysate. After 16 hours of solvent travel, the chromatograms were air-dried for 2–3 hours and then dipped into a silver nitrate solution similar to one used by Martin (5): it was prepared by dissolving 5.0 g. of silver nitrate in 3 ml. of pyridine, adding 197 ml. of acetone, and diluting the resulting solution 1:2 with acetone before dipping the chromatogram. After drying for a few minutes, the chromatogram was dipped into a sodium hydroxide reagent made by dissolving 0.60 g. of sodium hydroxide in 5 ml. of water and adding 95 ml. of ethanol. The reagent was diluted 1:2 with acetone before use. After the spots had developed for 15 minutes, the chromatogram was dipped into a 5% sodium thiosulfate solution containing 0.3% sodium sulfite. The chromatogram was held at room temperature until just damp (about 1 hour) and then dried at 105°–110°C. for 1 minute.

Quantitative estimation of sugars was made by measuring the optical density of the reduced silver spots with a Photovolt electronic densitometer (6). The cylinder lens was removed, and the entire oblong opening (4 cm.^{2}) was used with a Wratten No. 45 blue filter. Slopes of the curves of optical density vs. concentration varied from sugar to sugar. Data from arabinose may be cited as typical of the results obtained. Usually a difference of 0.12 in optical density values was equivalent to an increment of 2.5 γ of arabinose. The recovery of known

[2] Mention of firm names or trade products does not constitute endorsement or rejection by the U.S. Department of Agriculture over others of a similar nature not mentioned.

amounts of sugars was higher than 90%. Results are presented as percentage of sugar in the hemicellulose fraction.

With approximately 1-mg. quantities of air-dried cell walls, the sugars of the hydrolysate were determined by the Nelson modification of the Somogyi method as previously reported (10).

Nitrogen Determination. Total nitrogen was determined by the ultramicro-Kjeldahl method of Tompkins-Kirk (3).

Results

Qualitative Composition. Material chiefly obtained from cell walls by hydrolysis with 2% sulfuric acid was studied. This treatment affects the cell-wall hemicellulose and pectic substances. More drastic hydrolysis of the residues with 5% sulfuric acid yielded only glucose. This indicates that only cellulose remained unaffected by the original hydrolysis.

Arabinose and xylose were the main constituents of the hemicellulose fraction of all materials studied (Table I). Glucose was found in the hydrolysate of the cementing layer and in those of the cell walls of the endosperm, aleurone layer, and scutellar parenchyma.

TABLE I

ANALYSES OF THE HEMICELLULOSE FRACTIONS OF THE CEMENTING LAYER AND CELL WALLS IN THE CORN KERNEL

Material Analyzed	Sugars in Hemicellulose Fractions					Hemicellulose[a]
	Xylose	Arabinose	Glucose	Galactose	Uronic Acid	
	%	%	%	%	%	%
Pericarp	+	+	0	+	+	(50)[b]
Cross-cells	+	+	0	+	+	
Endosperm						
Aleurone layer	+	+	+	0	0	
Starchy portion[c]	35	34	31	0	0	21
Cementing layer	31	55	14	0	0	60
Germ						
Scutellar epithelium[d]	31	64	0	5	0	28
Scutellar parenchyma[e]	29	60	6	5	0	50

[a] Calculated from total weight of sugars in hydrolysate.
[b] Obtained by alkaline extraction (see reference 8).
[c] Average of nine analyses.
[d] Average of three analyses.
[e] Average of six analyses.

Galactose occurred in hydrolysates from the scutellar parenchyma and epithelial cell walls of the germ and also from the entire pericarp and cross-cells of the inner pericarp. Uronic acid was identified in the dilute acid hydrolysates from the pericarp and cross-cells, but not in those from any of the other cell walls. However, the fraction extracted from the pericarp with ammonium oxalate and hydrolyzed

with dilute acid contained no pectic material, as indicated by the absence of uronic acid.

Quantitative Composition. Because of the small quantity available, only 64 γ of cementing material were hydrolyzed. The hydrolysate yielded 21 γ of arabinose, 12 γ of xylose, and 5.3 γ of glucose (Table I). The recovered sugars were 60% of the air-dried weight of cementing material. Additional hydrolysis of the small amount of residue with 5% sulfuric acid yielded no additional carbohydrate material. This result confirmed an earlier observation that the cementing layer contains no cellulose (9).

Nitrogen determinations were made on two samples of cementing material; one weighing 35 γ had 30% protein (N × 6.25); the other sample weighing 103 γ contained 29% protein.

Cell walls of the scutellar parenchyma contained approximately 50% hemicellulose, those of the endosperm had only 21% (Table I), and scutellar epithelium cell walls averaged 28%. Previous work on the pericarp, with alkaline extraction methods, indicated a hemicellulose content of about 50% (8).

Of the four sugars identified in the hemicellulose fraction, xylose and arabinose represented the major components. About a third of the hemicellulose of the cell walls was xylose (29–35%), while the arabinose content varied from 34% (endosperm) to 64% (scutellar epithelium). The major difference in the composition of the hemicelluloses studied was the glucose content. Hemicellulose of the endosperm cell wall consistently showed a high glucose content in contrast to that of the cell walls of the scutellar epithelium, which had none. Only hemicellulose from cell walls of the scutellar parenchyma contained both glucose and galactose residues.

Discussion

Previous work showed that enzymatic separation of germ and endosperm of corn sections was effected by degradation of cell-wall pentosans and that tips of the epithelial cell walls of the scutellum were particularly susceptible to degradation (9). Consequently, the composition of the walls of the scutellar epithelium is of special interest. The present study shows that their hemicellulose content is relatively low. This fact is surprising in view of their comparative ease of degradation. Contamination with adjacent tissues is unlikely, since glucose would, in that case, have been found in the hydrolysate.

Examination of the epithelial cell walls with a polarizing microscope showed weak birefringence at the tips and a strong birefringence at the base. This difference in birefringence suggests a progressive in-

crease in cellulose content toward the base of the cells. Because of the method of isolation used, the wall material analyzed from these cells came mostly from the base, where the hemicellulose content may be low.

The hemicellulose from cell walls of the scutellar epithelium, unlike that from the other cells studied, was almost entirely pentosan (95%) and had the highest arabinose content of all hemicelluloses examined. Possibly the same chemical properties which render arabans more susceptible than other polysaccharides to acid hydrolysis may also account for the high enzyme susceptibility of these particular cell walls.

In the absence of pectic substances, it was assumed that the various types of cell walls studied were composed largely of hemicellulose and cellulose. Only qualitative determinations were made for cellulose (histochemical reactions, detection of birefringence, and further hydrolysis of residue left after removal of hemicelluloses). More work needs to be done, not only to elucidate the composition of the easily hydrolyzable hemicelluloses, but also to estimate the content of cellulose and other possible components such as protein and water-soluble polysaccharides.

The hemicellulose of the cementing layer is similar in composition to that of the endosperm cell walls. The presence of glucose, in particular, suggests strongly that the cementing material is of endosperm origin. This hypothesis appears to be reasonable since the germ in corn is known to develop partially at the expense of the endosperm. During the developmental period, there may be an infiltration of intercellular spaces over and around the epithelial cells by partial degradation products of the endosperm. On desiccation this material forms the carbohydrate-protein complex which becomes the so-called cementing layer between germ and endosperm.

Pectic substances are commonly regarded as important components of the primary cell wall and of the middle lamella, the bonding layer between adjacent cell walls. However, early work by Nanji and Norman (7) showed that only 0.59% of pectic material was present in corn kernels, and most of this was believed to be "in the middle lamella and cell wall of the aleurone layer." In the present study, as well as in previous work (9), no pectic materials were found in hydrolysates of any of the cells examined. Nor were pectic materials found in ammonium oxalate extracts of the pericarp. It has previously been shown, however, that corn pericarp contains a polyuronide hemicellulose with as much as 12% uronic acid (8). Possibly this has been mistaken by some workers for pectic material. Other workers

have reported low amounts of pectic substances in cereal grains. For example, Bishop *et al.* (1) found about 0.3% of pectic material (based on dry weight) in primary cell walls of the coleoptile of germinated oats.

Since pectic substances are virtually absent, the mechanism of tissue breakdown cannot involve pectic degradation. This concept agrees with previous results which showed that pectic enzymes had no obvious effect on cell walls of corn kernels (9). However, cell walls were degraded when treated with mixed pentosanase and cellulase in the absence of pectic enzymes.

The present work emphasizes the importance of hemicelluloses as structural components of cell walls of the corn kernel. Not only are they widely distributed in the kernel, but also they are easily solubilized, both enzymatically and chemically. They thus assume significance not only in controlling germ-endosperm separation, but also in weakening or degrading other cells within the kernel to aid in the release of starch, protein, and oil.

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THE DOUGH-MIXING BEHAVIOR OF GLUTEN AND OTHER FLOUR FRACTIONS TREATED WITH N-ETHYLMALEIMIDE[1]

D. K. MECHAM, H. A. SOKOL, and J. W. PENCE

ABSTRACT

The modification of dough-mixing behavior by N-ethylmaleimide (NEMI) appears to result from reaction with sulfhydryl groups in the less soluble portion of gluten as indicated by mixing curves of doughs that contained various flour fractions treated with NEMI.

After extraction of a hard red spring wheat flour with 0.5*M* sodium chloride, the gluten-starch residue retained a marked sensitivity to NEMI additions. The dialyzed solids from the salt extract did not affect this sensitivity. Also, NEMI-treated and untreated extracts had equal and small effects on mixing behavior of the residue in the absence of added NEMI.

After extraction with water, the gluten-starch residue differed markedly in mixing properties from the original flour or salt-extracted residue. Nevertheless, the water-extracted residue remained sensitive to the addition of NEMI. NEMI treatment of the water-soluble constituents did not modify their effects on mixing-curve characteristics.

The sustained interest of cereal chemists in the sulfhydryl groups in flours and doughs has been based largely on the possibility that "maturing" or "improving" agents affect physical properties of doughs by oxidation of the sulfhydryl groups (5). Recently it was suggested that sulfhydryl groups might be critically involved, also, in determining the mixing characteristics of a flour (3). This suggestion was based on the observed effects of some highly specific sulfhydryl-binding reagents on the mixing behavior of doughs. The present paper reports the results of efforts to determine whether the effects of N-ethylmaleimide (NEMI), in particular, on mixing behavior of doughs can be attributed to reaction with the sulfhydryl groups in a specific constituent or group of constituents of flour.

Observations of this kind seemed necessary because typical straight-grade flours contain only about 1 microequivalent (1×10^{-6} equivalent) of sulfhydryl per g. of flour, or, in a flour of 10% protein content, 10 microeq. of sulfhydryl per g. of protein. In contrast, the protein would contain about 100 or 125 micromoles of cystine per g. and 2400 of glutamic acid. Because of the very small proportion of sulfhydryl residues, measurement of reaction products of sulfhydryl groups and binding reagents in flour and doughs seemed likely to be most difficult, so that it would be advantageous to be able to fractionate the NEMI-treated dough and discard those portions not involved in the effect

[1] Manuscript received August 4, 1959. Presented at the 44th annual meeting., Washington, D.C., May 1959. Contribution from Field Crops Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany 10, Calif.

of NEMI. Furthermore, there is no reason to suppose that all the sulfhydryl groups of the gluten components, albumins, or globulins, even though they total only 1 microeq. per g. of flour, are equal in their effects on mixing properties or that they react equally readily with NEMI. Elimination of any of these constituents as participants would simplify study of the problem of accounting for the effects of NEMI on mixing characteristics.

Materials and Methods

Three hard red spring wheat flours were used. Two were milled commercially from Montana wheats, one in 1955, the other in 1958. Their protein contents, dry basis, were 14.8% and 15.6%, respectively. The third flour was milled experimentally from a composite of pure variety lots of Lee wheat grown in 1956; it contained 17.5% protein, dry basis. The flours were stored at − 10°F. (− 23.3°C.) in friction-top cans; small portions were removed as needed, allowed to equilibrate to room temperature at least overnight, and then used within 2 weeks.

Separations by Gluten Washing. In some exploratory experiments a conventional gluten-washing procedure was used. Doughs were mixed in a laboratory dough mixer until all flour was moistened and the mixture cohered, but dough development was avoided. Water and water solutions of NEMI were used to form doughs. The doughs were allowed to stand 30 minutes under water at room temperature, then were kneaded gently to separate starch and gluten in the usual way. The volume of water was restricted, however, to about 250 ml. per 100 g. flour. Doughs containing NEMI were very soft and sticky in the early stages of washing and had to be kneaded very carefully, but the gluten eventually cohered well enough to permit normal handling. The gluten was frozen in thin sheets by pressing onto blocks of solid carbon dioxide; and the frozen sheets were broken into pieces and lyophilized.

The starch suspension was centrifuged and the supernatant liquid retained. The starch precipitate was present as two distinct layers; the lower prime starch and upper tailings starch were separated with a spatula, and each was suspended in water. After centrifuging, the wash liquids were added to the first supernatant liquid, and the starch fractions were washed again in the same way. The final washings were discarded. The washed starch fractions were dried in a vacuum oven at 37°C.

The soluble constituents present in the wash liquids were recovered by vacuum concentration to a small volume in a rotating-flask evaporator followed by lyophilization. The various lyophilized fractions

were ground through a 40-mesh screen in a small Wiley mill and exposed to atmospheric humidity for at least 24 hours before nitrogen and moisture contents were determined.

The amounts of the fractions recovered and their nitrogen contents are given in Table I. Reconstituted doughs were prepared with the

TABLE I

FRACTIONS FROM 1955 HRS FLOUR BY GLUTEN-WASHING PROCEDURE

DOUGH	FRACTION	WEIGHT RECOVERED PER 86 G. FLOUR SOLID	TOTAL NITROGEN (DRY BASIS)
		g	%
Control	Gluten	13.3	13.7
	Prime starch	46.3	0.0
	Tailings starch	15.0	0.3
	Solubles	4.8	3.4
+ NEMI	Gluten	11.5	14.3
(6.6 mg. per	Prime starch	46.4	0.0
86 g. flour solids)	Tailings starch	14.5	0.5

fractions combined in proportion to the amounts recovered (dry weight) from control doughs. Thus 7.2 g. gluten, 25.1 g. prime starch, 8.1 g. tailings starch, and 2.6 g. solubles were combined to give 43.0 g. flour solids (equivalent to 50 g. flour solids, 14% moisture basis). These quantities were used to obtain mixing curves in a farinograph, using a 50-g. bowl. Although the proportions of fractions recovered from the NEMI doughs were slightly different (as were their nitrogen contents), the differences were considered to be relatively minor in view of marked differences in the mixing curves. Fractions from the NEMI-treated doughs therefore were substituted for control fractions on an equal-weight basis.

Extraction of Soluble Constituents. In addition to the gluten-washing procedure, flour fractions were obtained by the following procedure. Eighty-gram portions of flour were weighed into polyethylene bags and placed under vacuum to remove air; the vacuum was released with purified nitrogen, and the samples were let stand under nitrogen overnight. Eight hundred milliliters of water or other extractant were placed in a 1-qt. Mason jar and brought to freezing by holding the jar in a Dewar flask containing some powdered dry ice. During the cooling period, the extractant was flushed with a stream of purified nitrogen. An 80-g. portion of the flour then was transferred to the jar, and the jar was closed with an Osterizer blending assembly (John Oster Mfg. Co., Racine, Wisconsin).[2] These amounts of extractant and flour

[2] Mention of trade names or equipment does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

were chosen to minimize head space in the jar. The blender was run at full speed for 1 minute, then slowly for 4 minutes. (By allowing some ice to form when the extractant is cooled, the extraction can be maintained at 0°C. throughout the mixing.) The suspension then was centrifuged for 20 minutes in precooled cups; the centrifuge also was cooled by additions of powdered dry ice.

The extracts were decanted from the starch-gluten residue. NEMI-treated solubles were obtained by adding NEMI equivalent to 20 times the sulfhydryl content of the flour represented by the extract. The extracts were held 5.5 hours in an ice-water bath, then were transferred to cellophane dialysis tubing. Dialysis was carried out at 2° to 5°C. Dialyzed solids were recovered by placing the dialysis sacks in an air blast to evaporate most of the water, then lyophilizing.

The starch-gluten residues were frozen rapidly by spreading on dry ice blocks; frozen residues were broken into pieces, lyophilized, and ground through a 40-mesh screen.

Mixing curves were obtained with these fractions in a Swanson-Working recording mixer. In operating the mixer, a spring setting of 10 was used.

Also, mixing was stopped briefly after 1 minute and the dough was scraped down from the sides of the bowl. The absorption used was that required by the original flour to give a maximum of 500 Brabender units in a farinograph. Thirty-five grams of starch-gluten residue (14% moisture basis) were used in each dough. Additions of soluble constituents were made without reducing the quantity of starch-gluten residue.

The starch-gluten residues obtained by extracting flour with 0.5*M* sodium chloride contained about 2.1% of salt (dry basis), based on conductivity measurements of water extracts of the residues. Accordingly, sufficient starch-gluten residue (30.7 g. dry weight) was weighed out for each dough to provide 30.1 g. dry flour solids; this quantity contained 0.6 g. sodium chloride. Additional salt, 0.1 g., was added to give 2% salt (flour basis) in each dough. To doughs prepared from water-extracted flour residues, 0.7 g. salt was added.

Sulfhydryl analyses were carried out by amperometric titrations with silver nitrate (1) as adapted for use with flour samples (4).

Results and Discussion

Fractions by Gluten Washing. The fractions described in Table I were combined into reconstituted doughs and mixed in the farinograph. The composition of the doughs and some characteristics of the mixing curves are given in Table II. Substitution of prime starch or

TABLE II

EFFECTS OF N-ETHYLMALEIMIDE TREATMENT PRIOR TO GLUTEN WASHING ON FARINOGRAPH CURVE CHARACTERISTICS OF RECONSTITUTED DOUGHS [a]

Dough Constituents			Mixing Time to Maximum	Maximum Resistance	Decrease in Resistance 20 Minutes Past Maximum
Gluten	Prime Starch	Starch Tailings			
			minutes	*B.u.* [b]	*B.u.* [b]
Control	Control	Control	6.0	515	110
NEMI	Control	Control	3.3	510	140
Control	NEMI	Control	6.5	495	100
Control	Control	NEMI	6.3	495	95
NEMI	NEMI	NEMI	3.0	560	170

[a] All doughs contained "control" solubles. Water was added to give 62% absorption on a 14% moisture basis; this absorption with the original flour gave a curve maximum of 500 B.u.
[b] Brabender units.

tailings starch fractions from the NEMI-treated dough for the corresponding control fractions had no significant effects on the mixing behavior of the reconstituted doughs, but gluten from the NEMI dough decreased mixing time to 3.3 minutes. The latter value also is not significantly different from the 3.0-minute value for the dough containing gluten and both starch fractions from NEMI-treated dough.

Because the starch fractions were found not to be involved in the effect of NEMI on mixing, further studies were simplified, because gluten need not be separated from starch. This facilitated both the preparation of dough fractions and the mixing of reconstituted doughs.

The above observations and other similar exploratory experiments suggest that most of the effect of NEMI is due to its reaction with gluten sulfhydryl groups. However, the washing procedure that had been used for the separation of the dough fractions undoubtedly left appreciable amounts of globulins, and perhaps albumins, in the gluten. These soluble proteins then could have been involved in the NEMI effects. In addition, many of the sulfhydryl groups in the dough fractions were lost, presumably by oxidation, during the gluten-washing process. For example, one set of determinations indicated only one-fourth the sulfhydryl groups of the flour to be present in the fractions. To minimize these difficulties in subsequent work, extraction of solubles from flour suspensions was conducted at low temperatures and with nitrogen-flushed extractants.

Extractions with 0.5M Sodium Chloride. Gluten-starch residues and dialyzed solubles were prepared from the 1958 commercial flour, using a 10:1 (v/w) extractant-to-flour ratio as described above. Mixograph curves obtained with the original flour and its gluten-starch residue, without and with added NEMI, are shown in Fig. 1. Comparison of curves A and B shows the typical effects of addition of 1

microeq. of NEMI per g. flour. In the presence of NEMI (curve B), the mixing curve reaches a slightly higher maximum more rapidly and then breaks down very rapidly. The very narrow band beyond the maximum is quite typical; the contrast in width before and after breakdown is more pronounced in mixograph than in farinograph curves, however.

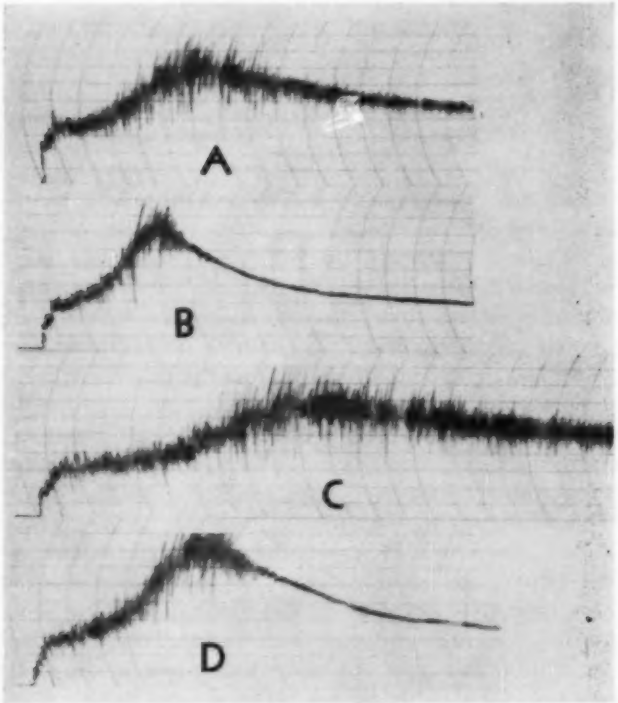

Fig. 1. Effect of extraction with 0.5*M* sodium chloride and of N-ethylmaleimide on mixograph curves of a hard red spring wheat flour (commercial milling, 1958). All doughs contained 35.0 g. flour or flour residue on a 14% moisture basis, 0.70 g. sodium chloride, and 22.8 ml. water. A, untreated flour; B, untreated flour plus 4.4 mg. N-ethylmaleimide; C, residue after extraction of flour with 0.5*M* sodium chloride; D, as C plus 4.4 mg. N-ethylmaleimide.

The residue from flour extracted with 0.5*M* sodium chloride (curve C) gave a longer mixing time to peak and somewhat slower breakdown than the original flour. In fact, the dough was tougher, more elastic, and in general stronger than that from the original flour. However, it is quite evident that the extracted flour retains a marked sensitivity to NEMI (curve D). The sulfyhydryl groups retained in the glu-

ten of the residue thus are sufficient to show the effect of NEMI, although the residue contains only about two-fifths of the sulfhydryl content of the original flour (0.48 vs. 1.12 microeq. per g.).

In contrast to the gluten-starch residue, the soluble constituents recovered by dialysis and lyophilization could not be shown to be involved in the effect of NEMI on doughs, as illustrated in Fig. 2. When

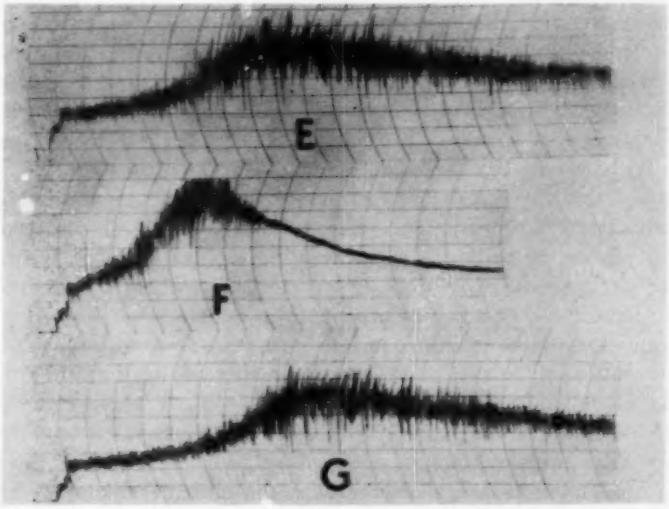

Fig. 2. Effect of additions of dialyzed extract solids and of N-ethylmaleimide on mixograph curves of gluten-starch residue extracted with 0.5*M* sodium chloride (hard red spring wheat flour, commercial milling, 1958). All doughs contained 35.0 g. flour residue or flour residue plus solubles on a 14% moisture basis, 0.70 g. sodium chloride, and 22.8 ml. water. E, salt-extracted residue plus dialyzed extract solids; F, as E plus 4.4 mg. N-ethylmaleimide; G, salt-extracted residue plus N-ethylmaleimide-treated dialyzed extract solids.

the control solubles were added to the gluten-starch residue, the mixing curve was little changed (compare curves C and E), and no diminution or enhancement of the effect of an NEMI addition was observed (compare the change from curve E to F with that from C to D). Also, treatment of the solubles with NEMI had, at most, a small effect on mixing curve characteristics (compare curves E and G).

The recovery of sulfhydryl groups in the residue and extract solubles, based on the actual weights of material recovered so that the value is minimal, was 52% of the original flour sulfhydryl groups—39% in the residue and 13% in the extracted solids. The largest losses probably occurred in the extracted solids, because of the prolonged dialysis required to remove salt. Also, the sulfhydryl content of ex-

tracted solids after NEMI treatment was 4.4 microeq. per g. compared to 5.7 microeq. per g. in the untreated extract solids. This relatively small difference suggests that in the untreated extract sulfhydryl groups were oxidized to nearly the same extent they were blocked by NEMI in the treated extract, and that only less reactive groups remain. Despite these unsatisfactory aspects of the observations, the marked sensitivity to NEMI retained by the 0.5*M* sodium chloride-extracted gluten-starch residue makes it unlikely that the more labile sulfhydryl groups in the extracted solids are of any considerable importance to the NEMI effect.

Extractions with Water. Mattern and Sandstedt (2) observed that the mixing characteristics of flours were altered markedly by extraction with water. The removal of water-soluble constituents increased mixing time; the constituents principally responsible appeared to be gliadinlike in composition, i.e., high in amide-N content. Mattern and Sandstedt noted also that extraction with sodium chloride solution did not alter mixing characteristics significantly. With the different flour and somewhat different procedures used in work described above, the gluten-starch residue extracted with 0.5*M* sodium chloride gave somewhat longer mixing times and slower breakdown than the original flour, as shown in Fig. 1. However, in agreement with Mattern and Sandstedt, these changes were relatively minor compared to those observed after water extraction of the same flour, as shown in Fig. 3.

Curve H, Fig. 3, was obtained with the gluten-starch residue from water-extracted flour. Nearly 1.25 hours of mixing were required before the residue particles cohered well enough to form a rather weak dough; then, however, a definite development took place. Breakdown of the dough likewise was very slow.[3] Addition of dialyzed water-extractable material shortened mixing time to about 25 minutes, but NEMI treatment of the extractable material appeared to have no significant effect. Mixing time was still about 23 minutes. The original mixing properties of the flour were not restored as completely as Mattern and Sandstedt (2) observed, but the effect was certainly pronounced and in the same direction. The incomplete restoration, in the present work, may arise in part from the removal of constituents of low molecular weight, by dialysis.

The recovery of sulfhydryl groups in the water-extracted flour residue and extract solubles, again based on the actual weights of material

[3] Appreciable evaporation of water from the dough occurred during this long mixing; control experiments with other doughs indicated that about 2.5 g. were lost in 1.5 hours of mixing, equivalent to 7.1% absorption. When a dough was mixed with the water-extracted gluten-starch residue with absorption reduced 10% below that used to obtain curve H, Fig. 3, development time was shortened to about 20 minutes. The long mixing time to maximum therefore might be attributed to the use of too high an absorption, after removal of an appreciable portion of the flour proteins. As shown later, however, the absorption used is not too high for rapid dough development when NEMI is added.

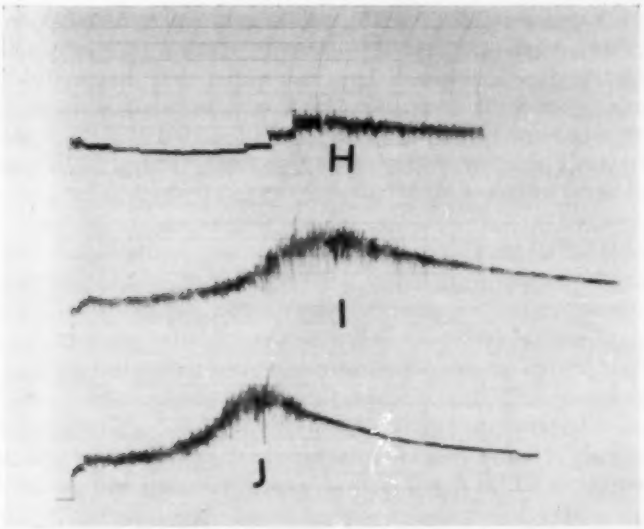

Fig. 3. Effect of extraction with water and of N-ethylmaleimide on mixograph curves of a hard red spring wheat flour (commercial milling, 1958). All doughs contained 35.0 g. flour residue or residue plus solubles on a 14% moisture basis, 0.70 g. sodium chloride, and 22.8 ml. water. H, residue after extraction of flour with water (the mixing curve for H only was recorded 1 minute of each 10 minutes of mixing; the total time represented on the chart is 2.5 hours); I, as H plus 4.4 mg. N-ethylmaleimide; J, water-extracted residue plus dialyzed water-extracted solids plus 4.4 mg. N-ethylmaleimide.

recovered, was 61% — somewhat higher than with 0.5*M* sodium chloride extraction. The distribution was markedly different, however, with 58% in the residue. The residue contained 0.75 microeq. per g.; the extract solids, only 1.4 microeq. per g., despite the fact that the flour nitrogen extracted by water (29% of the total) was nearly twice as much as extracted by 0.5*M* sodium chloride. NEMI treatment of the water extract did not lower the sulfhydryl content of the recovered solids; 1.5 microeq. per g. were found.

Because the dialyzed water extract of this flour contained 29% of the flour nitrogen, extensive changes in mixing behavior of the gluten-starch residue perhaps could be expected. The return toward original flour properties upon restoration of the extract solubles then could be expected also. The marked effect of NEMI on the gluten-starch residue was unexpected, however. As shown by curve I, Fig. 3, one microeq. per g. changed the dough from one that did not develop for more than an hour to one that coheres, develops to a peak in 10 minutes, and breaks down at a moderate rate. In the presence of added

NEMI, supplementation of the residue with dialyzed solubles had a moderate additional effect in shortening mixing time to peak resistance (curve J).

Important as the water-extractable constituents are to the normal mixing behavior of flours, these observations on the water-extracted gluten-starch residue and water-soluble constituents consistently indicate that the effects of NEMI are not exerted through the water-extractable material.

NEMI Treatment during Extraction. As described above, the gluten-starch residues from either 0.5*M* sodium chloride or water extraction show marked responses to NEMI additions. Nevertheless the recovery of sulfhydryl groups indicates that many of the more easily reacted sulfhydryl groups were lost during extraction and drying. In order to obtain residues in which these sulfhydryl groups might be expected to have combined with NEMI, extractions were conducted with 10 volumes of water and salt solutions, each of which contained a twofold excess of NEMI based on flour sulfhydryl content. Extraction conditions otherwise were as described above. A comparison of some of the mixing curves obtained with untreated and these treated residues is given in Table III.

The gluten-starch residue after extraction with NEMI-0.5*M* sodium chloride gave a curve nearly identical with that obtained from the

TABLE III

COMPARISON OF MICRO-RECORDING DOUGH MIXER CURVES OF 1958 COMMERCIAL FLOUR RESIDUES EXTRACTED WITH WATER OR 0.5*M* SODIUM CHLORIDE WITH AND WITHOUT ADDITION OF N-ETHYLMALEIMIDE

Dough Constituents	Mixing Time to Maximum	Resistance[a]	
		Maximum	10 Minutes Past Maximum
	minutes		
1. 0.5*M* sodium chloride-extracted residue; no NEMI present	10.5	670	530
2. 0.5*M* sodium chloride-extracted residue; NEMI present during extraction	10.5	610	420
3. As 2, plus 4.4 mg. NEMI in dough	7.2	680	510
4. Water-extracted residue, no NEMI present	about 90	330	330
5. As 4, plus 4.4 mg. NEMI in dough	10.3	600	360
6. Water-extracted residue, NEMI present during extraction	13.5	500	390
7. As 6, plus dialyzed water-extracted solubles	8.8	630	420
8. As 6, plus NEMI-treated dialyzed water-extracted solubles	8.8	620	410

[a] Units on the basis of 1000 for full width of chart.

residue extracted in the absence of NEMI, and the dough remained sensitive to further NEMI additions. The gluten-starch residue after NEMI-water extraction, in contrast, was changed in mixing behavior as compared to the residue after water-extraction. A dough was obtained similar to that from the water-extracted flour to which NEMI was added during dough mixing. The sulfhydryl contents of these residues were definitely lower than those of residues extracted in the absence of NEMI. Values were 0.34 microeq. per g. for the NEMI-0.5*M* sodium chloride sample (vs. 0.48 in the absence of NEMI), and 0.52 for the NEMI-water sample (vs. 0.75 in the absence of NEMI). Reaction of sulfhydryl groups thus was slightly more extensive in the water-extracted residue, but the difference does not seem sufficiently large to account for the differing response of the two preparations. Taken with the mixing results, the observations suggest that in the presence of salt the insolubility of the gliadinlike protein may protect certain critical sulfhydryl groups in the gluten complex from reaction with NEMI during extraction of the flour suspension.

Behavior of Other Flours. Observations with fractions prepared from the flour from Lee wheat are summarized in Table IV. The resi-

TABLE IV

COMPARISON OF MICRO-RECORDING DOUGH MIXER CURVES OF LEE FLOUR AND GLUTEN-STARCH RESIDUES [a]

DOUGH CONSTITUENTS	MIXING TIME TO MAXIMUM	RESISTANCE [b]	
		Maximum	10 Minutes Past Maximum
	minutes		
1. Untreated flour	8.0	700	480
2. As 1, plus 4.4 mg. NEMI	5.5	770	340
3. 0.5*M* sodium chloride-extracted residue	14.5	760	560
4. As 3, plus 4.4 mg. NEMI	8.5	810	380
5. Water-extracted residue	... [c]	...	...
6. As 5, plus 4.4 mg. NEMI	21.5	480	350

[a] 35 g. flour or gluten-starch residue and 67% absorption on a 14% moisture basis.
[b] Units on the basis of 1000 for full width of chart.
[c] After about 1.5 hours the residue particles cohered to some extent but showed little indication of dough development.

dues recovered after 0.5*M* sodium chloride and water extraction differed in mixing behavior in the same manner as those from the 1958 commercial HRS flour. Their response to NEMI additions also was similar.

The Lee flour contained 0.97 microeq. sulfhydryl per g. Extraction with 0.5*M* sodium chloride removed 14% of the flour nitrogen, and the residue contained 0.59 microeq. sulfhydryl per g. In contrast, extraction with water removed 48% of the flour nitrogen, but the residue contained 0.78 microeq. sulfhydryl per g. The retention of sulfhy-

dryl groups in the residues was higher with this flour than with the 1958 flour. The differences between salt and water extractions in sulfhydryl content and amount of nitrogen extracted are similar, however.

Doughs made up of a commercial gum gluten and starch also showed the typical effect of NEMI on mixing curve characteristics.

General Discussion

The results reported above consistently point to the gluten fraction of flour as the medium through which NEMI additions exert their effect on dough-mixing properties. Despite the presence of reactive sulfhydryl groups in the soluble constituents, treatment of the solubles with NEMI could not be demonstrated to change dough-mixing characteristics. In contrast, all the gluten or gluten-starch preparations were sensitive to this addition of NEMI. In the case of the residues after extraction with salt solution, the effect of the NEMI additions was deleterious. With water-extracted residues, however, small additions of NEMI appeared to compensate to some extent for the changes brought about by removal of water-soluble substances. The proportion of protein extracted by water was high with the two flours used. In our experience this is typical of HRS flours. The behavior of other types of flours has not been investigated in this regard.

Comparison of the results from water- and salt solution-extracted flours suggests first that the effects of NEMI are exerted by reaction with the nongliadin, or at least nonwater-dispersible, portion of the gluten complex. A somewhat different suggestion also can be offered, however. The appearance of mixing curves of doughs to which NEMI has been added is similar to that of doughs to which an excess of gliadin has been added, as published by Mattern and Sandstedt (2). In both cases, a fairly short mixing time to maximum resistance is observed, followed by a sudden and pronounced narrowing of the recorded curve as mixing is continued. This suggests that NEMI reacts with the water-extracted residue to liberate some gliadinlike material and so tends to restore mixing properties of the flour residue to its original pattern. The sulfhydryl groups responsible for the NEMI effect then could occur in either the nongliadin or the liberated gliadinlike fractions.

Further investigations, particularly chemical studies, to determine the site of the NEMI-sulfhydryl reaction product in doughs mixed with added NEMI are needed to clarify the mechanism of the changes produced by NEMI. Information of this kind may help to provide an understanding of the chemical basis for the varied mixing characteristics of different flours.

Acknowledgment

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THE BROMATE REACTION IN DOUGH

I. Kinetic Studies[1]

W. Bushuk and I. Hlynka

ABSTRACT

The reaction of the bromate ion in nonfermenting doughs was studied under various conditions by determining residual bromate by the amperometric titration method. The over-all reaction is characterized by two distinct phases: a rapid initial reaction that occurs almost completely during mixing, and a slower, secondary reaction that proceeds at a constant rate. Reaction orders with respect to bromate concentration were found to be about 1.0 and 0.7 for the initial and the linear reactions respectively. Rates of both phases increase with increasing flour concentration and decreasing pH. The activation energies obtained for the range 10° to 50°C. are 1.48 and 10.16 kcal. per mole for the initial and linear reactions, respectively.

The effect of bromate on the physical properties of dough has attracted the attention of cereal chemists for a long time, and papers dealing with various aspects of the bromate reaction in dough have been published (3,6,8). However, no plausible mechanism for this reaction has so far been worked out.

[1] Manuscript received July 23, 1959. Paper No. 178 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg 2, Canada.

It has been demonstrated recently in this Laboratory that the amperometric titration can be used to determine residual bromate ion in dough (1). This procedure was also used successfully to follow the progressive disappearance of bromate in normal and modified doughs (1,2). It seemed desirable to continue the kinetic studies of the bromate reaction in dough, as they offer a reasonable approach to formulation of a mechanism for the reaction. Such information might be useful in explaining the action on the physical properties of dough of potassium bromate and similar agents.

This paper deals with the effects on the reaction rate in nonfermenting doughs of a number of factors normally investigated in kinetic studies, namely, bromate concentration, flour concentration, hydrogen ion concentration, ionic strength and temperature.

Materials and Methods

An untreated, straight-grade flour, milled commercially from hard red spring wheat, was used for all studies. Starch, used as a diluent in one experiment, was separated from the same flour. The flour was mixed into a dough and worked up by hand in a beaker of tapwater, and excess water was decanted after centrifuging. Resuspending in water and centrifuging for 15 minutes at 2,000 r.p.m. served to separate squeegee and granular starch. The latter was air-dried to a moisture of 12.7%. The flour and starch had protein contents of 13.2% and 0.21% and ash contents of 0.46% and 0.15% (14% moisture basis).

Some minor modifications were made in the methods (1) previously used for preparing and handling the doughs. Prior to mixing, the flour was evacuated and stored overnight under nitrogen in a vacuum desiccator. Air was removed from the liquids used by bubbling nitrogen through for 5 minutes. Doughs were mixed for 5 minutes in the GRL mixer (4) under nitrogen to 60% absorption, except where indicated otherwise. Gas analyses showed that the atmospheres under which the flour was stored and mixed contain small amounts of residual oxygen. The effect of this oxygen on the bromate reaction was neglected. During an experiment, the dough was stored at 30°C., except in the temperature study, at about 85% relative humidity. In the temperature study, the dough was mixed and stored at 10°, 20°, 40°, and 50°C., in addition to the normal temperature of 30°C.

Residual bromate was determined immediately after mixing and at hourly intervals up to 5 hours. The extraction procedure was essentially the same as outlined previously (1), except that the quantity of dough was reduced to 20 g. and extraction liquid comprised 90 ml. of water, 12.5 ml. of 1.04*N* zinc sulfate solution, and 12.5 ml. of 0.65*N*

sodium hydroxide solution. After dispersion in a Waring Blendor for 3 minutes at low speed and centrifugation, duplicate 20-ml. aliquots were titrated (1).

The potassium bromate concentration in mg. per kg. of dough (60% absorption) is obtained from the results for 20-ml. aliquots by multiplying the difference between the blank and sample titrations in ml. by 9.046.[2] The calculation of this factor involves measurement of the total volume of the dispersion containing 20 g. of dough and the 115 ml. of extraction liquid. For 60% absorption this volume is 130 ml.

Results and Discussion

Treatment of Results. In earlier papers (1,2), results were reported on a flour basis in p.p.m. of potassium bromate and were plotted in terms of residual bromate. For this study, it was found more convenient, and analogous to practices in solutions, to report results on a dough basis in mg. of potassium bromate per kg. and to plot them as bromate reacted. In the new terminology, 18.75 mg. of potassium bromate per kg. of dough of 60% absorption is equivalent to 30 p.p.m. of potassium bromate in a flour of 14% moisture content.

The calculations used in processing the data are illustrated in Table I and Fig. 1, which show results for a typical experiment with 18.75 mg. per kg. initial bromate in a dough of 60% absorption.

TABLE I

DATA FOR A TYPICAL EXPERIMENT AT 18.75 MG. PER KG. POTASSIUM BROMATE AND 60% ABSORPTION

Reaction Time	Sample Titration	Blank[a] − Sample V	Residual Bromate V × 9.046 = K	Bromate Reacted 18.75 − K
hours	*ml*	*ml*	*mg/kg*	*mg/kg*
0	...	...	18.75	0
0.08[b]	3.11	1.92	17.37	1.38
1	3.35	1.68	15.20	3.55
2	3.43	1.60	14.47	4.28
3	3.57	1.46	13.21	5.54
4	3.73	1.30	11.85	6.90
5	3.86	1.17	10.58	8.17

[a] Blank titration = 5.03 ml.
[b] First determination at approximately 3 minutes after mixing.

Figure 1 shows the amount of bromate reacted as a function of reaction time. It seems that the over-all reaction investigated consists of two relatively distinct phases, namely, a rapid initial reaction which occurs almost entirely during mixing, and a slower secondary reaction

[2] Potassium bromate, mg. per kg. of dough = (blank, ml. − sample, ml./1000) × (N of iodate) × V(ml.)/20 × 167.01/6 × 1000/20 × 1000 = (blank, ml. − sample, ml.) × 9.046 for N = 0.001 and V = 130 ml.

in which the disappearance of bromate varies approximately linearly with reaction time. In this paper, the reaction will be characterized by the amount of bromate reacted in the initial reaction and the rate of the linear reaction. The amount of bromate reacted was adopted as an index of the initial reaction, since it is not possible to measure the initial rate with any degree of accuracy. These parameters are determined as shown in Fig. 1.

Effect of Bromate Concentration. Table II summarizes the results of experiments at 30°C. and 60% absorption for different initial bromate concentrations. The concentrations of potassium bromate used are given in the first column in p.p.m. of flour and in the second in

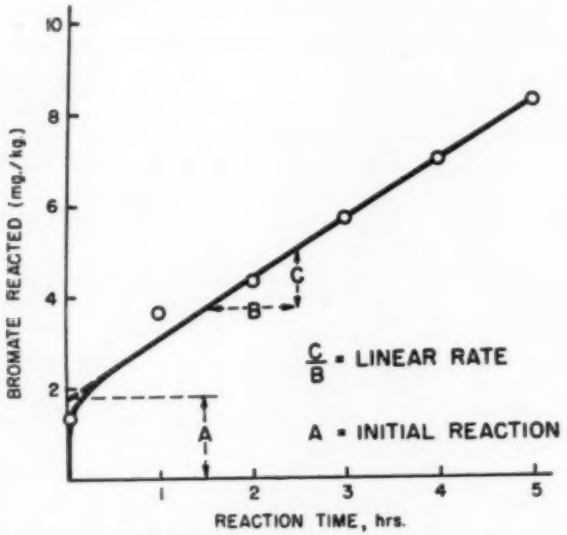

Fig. 1. Bromate reacted as a function of reaction time at 30°C. and 60% absorption, with initial potassium bromate concentration of 18.75 mg. per kg. of dough.

TABLE II

RESULTS WITH VARIOUS INITIAL POTASSIUM BROMATE CONCENTRATIONS

(Absorption = 60%)

BROMATE CONCENTRATION		INITIAL REACTION	LINEAR RATE
ppm flour	*mg/kg of dough*	*mg/kg*	*mg/kg/hour*
5	3.12	0.50	0.40
10	6.25	1.10	0.60
15	9.37	0.90	0.86
20	12.50	1.30	0.97
30	18.75	1.80	1.28
40	25.00	2.40	1.30

mg. per kg. of dough. The third and fourth columns give the amounts of initial reaction and the linear rates respectively.

The relation between the amount of initial reaction and the initial bromate concentration seems to be linear, whereas the rate of the linear reaction increases with increasing bromate concentration according to a relation that is definitely curvilinear (Fig. 2). A similar curvilinear relation results if the linear rate is plotted against initial bromate concentration corrected for the amount of initial reaction.

Determination of the reaction order with respect to bromate concentration, by plotting the logarithm of the rate against the logarithm of the concentration, gave an order of about 1.0 for the initial reaction and 0.7 for the linear reaction at low bromate concentrations. The order of 0.7 appears to decrease as the bromate concentration exceeds 18.75 mg. per kg. In making the logarithmic plot for the initial reaction it was assumed that the rate was directly proportional to the amount reacted.

Similar analysis of the data for the linear reaction for four flours published by Cunningham and Anderson (1) gave a reaction order of the same magnitude for three of the flours; the order for the fourth flour was about 0.4.

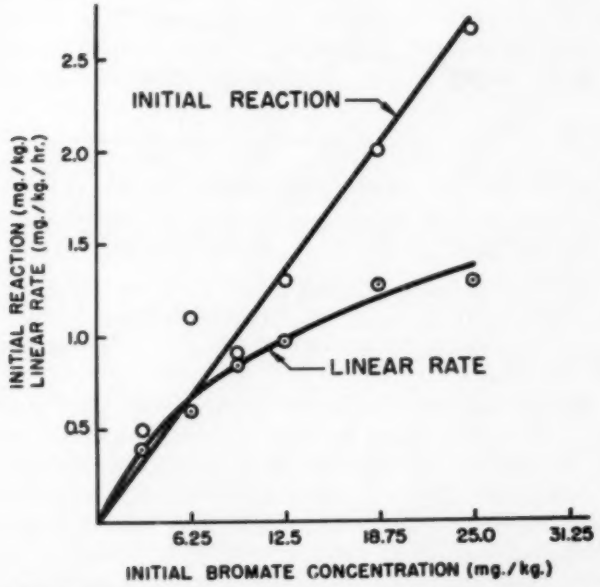

Fig. 2. Amount of initial reaction and rate of linear reaction at 30°C. and 60% absorption, as a function of initial potassium bromate concentration.

Effect of Flour Concentration. In an attempt to vary the concentration of the component of flour which reacts with the bromate ion, it was necessary to study the rate of bromate reaction in doughs containing different amounts of water (various absorptions). This created the problem of whether the bromate concentration should be kept constant on the basis of total dough weight or on the basis of the amount of liquid used to mix the dough. The former method was adopted because it seemed to be more in line with the method adopted initially for expressing bromate concentration.

The results that were obtained for the range of absorptions that could be used in the GRL mixer, with the initial bromate concentration of 18.75 mg. per kg., are plotted in Fig. 3. Although the total varia-

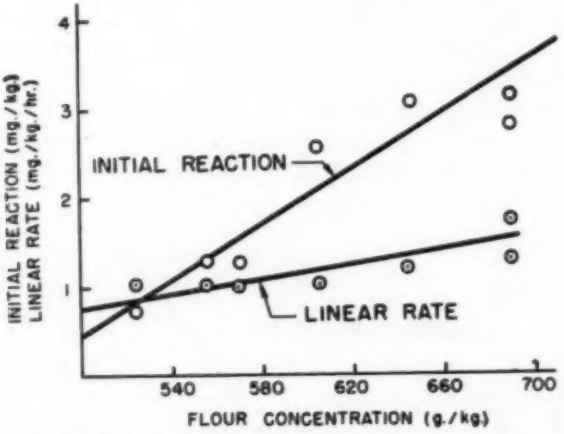

Fig. 3. Amount of initial reaction and rate of linear reaction at 30°C., as a function of flour concentration obtained by varying the absorption.

tion in flour concentration was only about 1.5 times, it seems that, in spite of the scatter, both the amount of initial reaction and the linear rate increase approximately linearly but at slightly different rates with flour concentration.

The effective concentration of the reactive component of the flour can also be varied by diluting the flour with the nonreactive component(s). In this manner the maintenance of a constant bromate concentration is simplified, since a constant absorption can be used. Experiments were therefore made with doughs from flour samples which were diluted by additions of starch.

Figure 4 shows the results of two levels of starch used with initial bromate concentration of 18.75 mg. per kg. and 60% absorption. It is

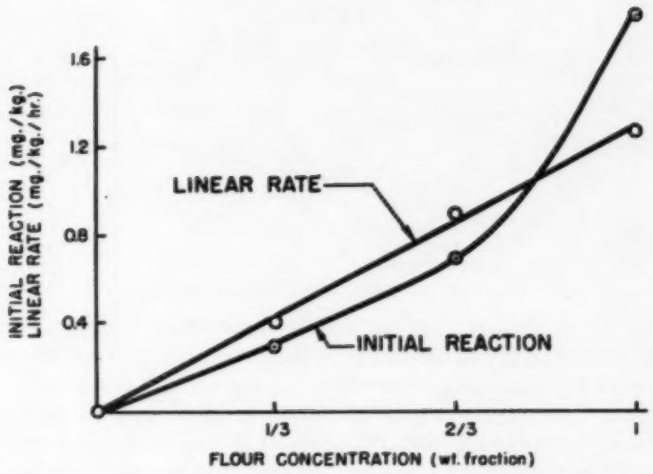

Fig. 4. Amount of initial reaction and rate of linear reaction at 30°C. and 60% absorption, as a function of flour concentration obtained by diluting the flour with starch.

apparent that both the amount of initial reaction and the linear rate increase with increasing flour concentration. These data indicate also that of the components of flour, it is not the starch that reacts with the bromate. Further studies of the bromate reaction in doughs mixed from various flour fractions may lead to a more specific localization of the reaction site or group.

Effect of Hydrogen-Ion Concentration and Ionic Strength. A study of the effect of pH on the bromate reaction in dough was made for two reasons. The first is that stoichiometric equations for oxidations by the bromate ion involve hydrogen ions, so that the pH dependence of the bromate reaction should give some indication of whether bromate reacts in dough as an oxidizing agent. The second reason is that a marked pH effect on the bromate reaction has been recently demonstrated by structural relaxation studies (5), and it was of interest to verify these observations by a different method. Studies on the effect of ionic strength can be useful in identifying certain types of ionic reactions.

Table III gives the results of experiments on doughs of pH varying from 4.2 to 8.7 as measured by the Beckman Zeromatic pH meter equipped with rugged electrodes. The pH of the dough was varied by adding predetermined quantities of hydrochloric acid or sodium hydroxide solution to the liquids used to mix the doughs. The actual amounts used are shown in Table III. The initial bromate concentra-

TABLE III

EFFECT OF pH ON THE BROMATE REACTION
(Initial potassium bromate = 18.75 mg./kg.; absorption = 60%)

pH	LIQUIDS, 90 ML. (+ 30 ml. of Bromate Solution)	INITIAL REACTION	LINEAR RATE
		mg/kg	*mg/kg/hour*
4.2	0.04*N* HCl	3.40	1.83
4.8	0.02*N* HCl	2.20	1.46
5.8	Distilled water	1.80	1.28
7.2	0.04*N* NaOH	1.80	1.10
8.7	0.08*N* NaOH	1.80	0.50

tion used was 18.75 mg. per kg. and the absorption was 60%. The reaction curves at pH values other than the normal pH of the dough (5.8) were of the same general shape as the normal curve.

Figure 5 shows the amount of initial reaction and the linear rate as a function of dough pH. The amount of initial reaction increases markedly with decreasing pH on the acid side but seems to be independent of pH on the alkaline side of the pH of a normal dough. The rate of the linear reaction varies inversely with pH over the range investigated. These findings are in general agreement with the hypothesis that bromate reacts as an oxidizing agent in dough and with the conclusions made by Hlynka and Chanin (5) based on the effect of pH on the rheological properties of dough.

Another possible explanation for the marked pH effect is the effect of hydrogen ions on the ionization of the reactive group of the flour. At present there is no evidence for or against this hypothesis.

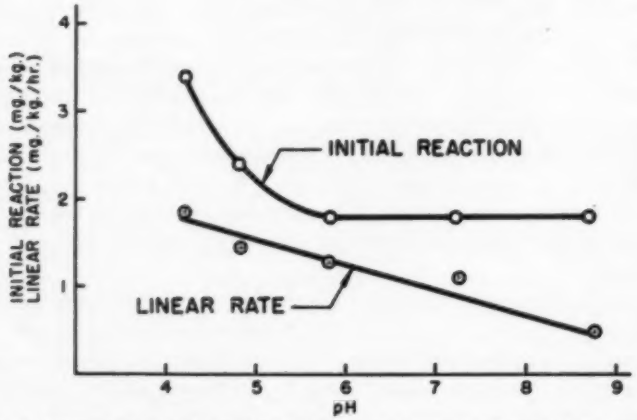

Fig. 5. Amount of initial reaction and rate of linear reaction at 30°C. and 60% absorption, as a function of dough pH.

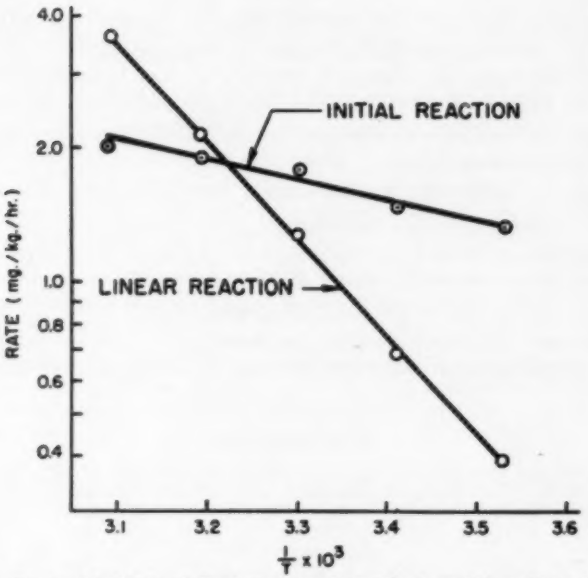

Fig. 6. Rates of initial and final reaction (logarithmic scale) at 60% absorption, as a function of the reciprocal of the absolute temperature.

The bromate reaction was not affected by variations of ionic strength by additions of up to 1.8% (flour basis) of sodium chloride. Accordingly, it appears that the bromate ion reacts with a neutral group rather than with another ion.

Effect of Temperature. Figure 6 shows the effect of temperature, plotted in the usual manner, on the two phases of the bromate reaction covering a temperature range of 10° to 50°C. Both the amounts of initial reaction and the linear rates plotted against 1/T give exceptionally good straight lines.

The activation energies obtained from the slopes of the lines in Fig. 6 are 1.48 kcal. per mole and 10.16 kcal. per mole for the initial and the secondary phases respectively. In the calculation of the activation energy for the initial reaction, it was assumed that the rate of the initial reaction is directly proportional to the amount of initial reacton, as was done in the analysis of the effect of bromate concentration.

General Discussion

The evidence presented in this paper suggests that the disappearance of bromate during the so-called initial reaction is real and not an experimental artifact as implied by Lee, Tkachuk, and Finlayson

(7). The two phases of the over-all bromate reaction are affected by the various factors examined in a similar way; accordingly, it is probable that the linear reaction may depend on the initial reaction. The activation energy of 1.48 kcal. per mole for the initial reaction suggests that the rate of this reaction is controlled by a physical process such as diffusion (mixing).

A reaction order of 0.7 with respect to bromate concentration for the linear reaction is not inconsistent with the fact that the over-all order appears to be zero. Such results are possible for extremely slow reactions. The activation energy of 10.16 kcal. per mole obtained for the linear reaction is in the range of values for reactions involving the breakage and formation of covalent bonds, although it does not rule out the possibility that activated diffusion might be the rate-controlling factor.

Acknowledgment

The authors wish to make grateful acknowledgment to D. K. Cunningham, formerly of the Grain Research Laboratory and now with The Pillsbury Company, who did some preliminary work on the study reported in this paper.

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OBSERVATIONS ON THE REACTIVITY OF SULFHYDRYL GROUPS IN WHEAT FLOUR[1]

H. A. Sokol, D. K. Mecham, and J. W. Pence

ABSTRACT

In flour samples dispersed in urea solutions (pH 7.0–7.5), sulfhydryl groups reacted faster and more extensively with mercuric chloride and N-ethylmaleimide than with iodoacetamide; with fourfold excesses of these three reagents, 100, 70, and 10%, respectively, of the total sulfhydryl groups reacted in 20 minutes. Very little or no effect of potassium bromate on the sulfhydryl content of doughs mixed in air was found at normal bromate dosages with straight-grade flours. However, with a fifth-break flour, a small decrease in sulfhydryl content resulted from bromate additions to fermented and nonfermented doughs. During prolonged mixing in air, about half the sulfhydryl groups of a spring wheat flour were lost. Additions of an anionic detergent of the alkyl sulfate type did not affect this loss, although the expected stabilization of a recording mixer curve was observed.

The action of flour improvers has often been postulated as an oxidation of sulfhydryl groups in flour proteins to form intermolecular disulfide bonds. These linkages would then account for increased toughness and gas retention in doughs and larger loaf volume in bread. On this basis, sulfhydryl-disulfide relationships in wheat products have interested cereal chemists for many years. The available information has been reviewed at intervals (1, 12, 13) so that no elaboration is needed here. Evidence also has been presented that sulfhydryl groups may be a factor in determining the mixing characteristics of doughs (10). Nevertheless, the participation of sulfhydryl groups as a critical factor in either dough mixing or the action of oxidizing improvers has not been positively demonstrated.

A principal obstacle has been the low level at which sulfhydryl groups occur in flours and the consequent analytical difficulties in tracing changes in sulfhydryl content. However, the silver titration method of Benesch, Lardy, and Benesch (3) has been applied to flour and flour constituents (9, 13), and found sufficiently sensitive and precise to encourage a study of the reactivity of sulfhydryl groups in flour. Before devoting particular attention to either dough oxidation or mixing in relation to sulfhydryl changes, a survey was made of the general reactivity of sulfhydryl groups in flour, including their reactivity with various specific sulfhydryl reagents. These findings will be reported here.

[1] Manuscript received June 1, 1959. Presented in part at the annual meeting, Cincinnati, Ohio, April 1958. Contribution from Western Regional Research Laboratory, one of the laboratories of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany 10, California.

Materials and Methods

The flours used were selected to represent quite different types. They were described in a previous publication (13).

The iodoacetamide (IAC) was a laboratory preparation; *p*-chloromercuribenzoate (PCMBA) was from Sigma Chemical Co., 3500 DeKalb Street, St. Louis, Mo.[2]; N-ethylmaleimide (NEMI) from Mann Research Laboratories, 136 Liberty St., New York 6, N. Y.; and Duponol "R" (mixture of fatty alcohol sulfates) from E. I. du Pont de Nemours & Co., Wilmington 98, Dela.

The method employed for the determination of sulfhydryl groups in flour, dough, and flour fractions was essentially the silver amperometric titration of Benesch, Lardy, and Benesch (3). Precautions to be observed and the particular procedures followed in applying the method to flour have been described (13). Dispersion of samples and titration at 2°C., addition of ethylenediaminetetraacetate (EDTA), and titration of dispersions rather than extracts or digests were found to be required for maximum recovery of sulfhydryl (13). In addition, each result was obtained from at least three individual titrations.

Difficulties and inconveniences were encountered in applying the method directly to doughs and flour-water slurries. Well-mixed doughs required prolonged exposure (up to 16 hours) to the titrating medium for complete dispersion, and, after such an interval, the sulfhydryl titer of the sample was reduced almost to zero. Unless freshly prepared for each titration, slurries also showed a significant decline in sulfhydryl content in successive titrations.

To minimize these losses, doughs and slurries were dried by lyophilization. Doughs were frozen rapidly by spreading them in thin layers on dry ice, and slurries were shell-frozen in balloon flasks in a dry ice and acetone bath. After lyophilization, grinding, and moisture equilibration, the dried doughs and slurries were handled like ordinary flour samples.

A small loss of sulfhydryl content occurred during the lengthy lyophilization. This loss was detected by a comparison of flour-water slurries with lyophilized portions of the same slurries. The slurries were made by blending three parts by weight of flour with four parts of distilled water in a food blender. After a few minutes of mixing, a portion was immediately frozen and lyophilized, and another portion of the slurry was immediately titrated. Comparisons of the amounts of titrant used for three separate slurry preparations and their lyophilized counterparts are given below:

[2] Mention of trade names, equipment, or suppliers does not constitute endorsement by the Department of Agriculture over others not mentioned.

Sample	*Dry Weight of flour*	*0.001N Silver Nitrate*
	g	*ml/g dry flour*
Slurry	0.533	0.71
After lyophilization	0.535	0.66
Slurry	0.675	0.71
After lyophilization	0.677	0.62
Slurry	1.032	0.64
After lyophilization	1.035	0.61

Sulfhydryl contents of doughs given in this paper, therefore, are probably 5 to 10% low. However, differences between samples should be reflected reasonably well, since all were dried by the same procedure. No loss of sulfhydryl groups has been observed in flours during storage for a month at room temperature (about 24°C.), although in one instance the sulfhydryl content of a flour decreased slightly in 2 months at room temperature. Lyophilized doughs are stable for at least 1 week at room temperature. No changes have been observed in flours stored at −18°C. for at least 2½ months.

Results and Discussion

Reactivity with Specific Sulfhydryl Reagents. The reactivity of flour sulfhydryl groups with two organic reagents (NEMI and IAC) and with mercuric ions was compared under the conditions previously found to permit titration of maximum amounts of sulfhydryl groups with silver – that is, in urea-containing suspensions at 2°C. Results are presented in Table I. Reaction proceeded much more readily with NEMI and mercuric chloride than with IAC; the maximum extents of reaction observed were, respectively, 90, 100, and 30%. The rate

TABLE I

REACTION OF N-ETHYLMALEIMIDE, MERCURIC CHLORIDE, AND IODOACETAMIDE WITH SULFHYDRYL GROUPS IN A COMMERCIAL HARD RED SPRING FLOUR[a]

REAGENT	TIME	SULFHYDRYL BLOCKED		
		CONCENTRATION OF REAGENT (equiv. per equiv. sulfhydryl)		
		2	4	8
	minutes	%	%	%
NEMI	20	40	70	70
	90	90	90	90
IAC	20	10	10	10
	90	30	30	30
Mercuric chloride	20	100[b]		

[a] Flour sample suspended in 6.4 *M* urea, 0.1 *M* tris (hydroxymethyl) aminomethane, pH 7.5. Values are averages of three titrations.
[b] No EDTA used in titration.

of reaction of sulfhydryl groups with IAC was not changed by increasing the IAC concentration; whereas a change was noted with increased NEMI concentrations for the 20-minute reaction time. IAC is known to be a slow-reacting sulfhydryl reagent. Lontie and Beckers (8) showed that reaction times with IAC up to 24 hours were necessary to get maximum sulfhydryl titers for ovalbumin; in addition, Olcott and Fraenkel-Conrat (11) indicated that IAC does not always react with sulfhydryl groups. They point out that tobacco mosaic virus can be inactivated by IAC with no decrease in sulfhydryl content. It may be that the sulfhydryl groups must be in a highly activated state to react with IAC. The number of such groups may be only a small fraction of the total number at any particular time. This would account for the inability of increased IAC concentrations to affect the sulfhydryl content of the dispersions (Table I).

In reactivity tests with flour similar to those made with NEMI and IAC, PCMBA as the specific reagent gave unsatisfactory results, apparently because of reversibility of the PCMBA-sulfhydryl reaction. This was indicated by rounded amperometric titration curves having no definite end point. They improved in shape only when large excesses of PCMBA were added. The results with NEMI provide evidence, in addition to that presented previously (13), that the specificity of the silver-tris [tris (hydroxymethyl) aminomethane] titration for sulfhydryl groups is, at least, very largely retained in the presence of nonprotein flour constituents, since the silver titer is reduced by 90%. It seems most likely that the few residual sulfhydryl groups are in such positions that they cannot react with NEMI. An inability of excessive amounts of NEMI to eliminate sulfhydryl titers completely at pH 10.4 has been reported by Bloksma (5) and, at a lower pH, by Matsumoto and Hlynka (9).

Effect of Potassium Bromate. Bloksma (4) found no changes in the sulfhydryl content of glutens separated from doughs to which bromate had been added. In contrast, Matsumoto and Hlynka (9) found a definite lowering of the sulfhydryl content of gluten proteins separated from doughs, mixed in the absence of oxygen, to which bromate or iodate was added. Observations made in the present study on whole doughs, rather than glutens, are given in Table II. The doughs (50 g. flour, 35 ml. distilled water, and the indicated amounts of yeast and potassium bromate) were mixed at 29°C. for 10 minutes in a farinograph and allowed to stand for 3 hours at room temperature before freezing and lyophilization. Titrations were made shortly thereafter. No effect of bromate was found on the sulfhydryl content of doughs from Lee and Idaed variety flours. However, with the fifth-break flour,

TABLE II

SULFHYDRYL CONTENT OF FERMENTED, NONFERMENTED, AND BROMATED DOUGHS

FLOUR	NO YEAST		2.5% YEAST	
	Bromate	Sulfhydryl Content	Bromate	Sulfhydryl Content
	ppm	*μeq/g*	*ppm*	*μeq/g*
Lee[a]	0	0.68	0	1.17
	30	0.69	20	1.16
Idaed[a]	0	0.64	0	1.05
	50	0.64	40	1.02
Fifth-break[a]	0	0.96	0	1.34
	50	0.76	50	1.28

[a] The sulfhydryl contents of Lee, Idaed, and fifth-break flours were 1.04, 0.87 and 1.30 microequivalents per gram, respectively.

small decreases in sulfhydryl content as a result of potassium bromate additions were observed. The fifth-break flour has a very high protein content and a proportionately high sulfhydryl content; it cannot be considered a typical bread flour. However, this flour also has a very high baking response to bromate, in contrast to the Lee and Idaed flours, so that the decrease in sulfhydryl content is consistent with the correlation between sulfhydryl groups and bromate response first postulated by Sullivan (16).

Sulfhydryl Contents of Materials Extracted by Petroleum Ether. Sulfur-containing lipoproteins are known to be present in petroleum ether extracts of flours (2). Matsumoto and Hlynka (9) found that the sulfhydryl content of the petroleum ether extract of hard red spring flour was negligible in relation to the sulfhydryl content of other fractions. Similar results were obtained here. The averages of four to six individual determinations per sample were as follows:

Flour	*Sulfhydryl Content*	
	Untreated	*Extracted*
	μeq/g flour	*μeq/g flour*
Cookie flour (commercial)	0.66	0.66
Lee	0.89	0.86
Idaed	0.86	0.88

Effects of Added Detergent. An increase in the mixing stability of flours from addition of anionic detergents has been reported by Sullivan (15) and by Swanson *et al.* (17). This effect is opposite to that of NEMI and other sulfhydryl-blocking reagents (8) and suggests that the detergents act by protecting sulfhydryl groups during mixing. In addition, Klotz (7) and Van Scott and Flesch (18) reported high sulfhydryl titers for bovine serum albumin and fibrous proteins when

detergents were used as denaturing agents.

To determine whether the sulfhydryl content of doughs would be altered by inclusion of a detergent, doughs were prepared from Lee variety flour in a farinograph and sampled after 35 minutes of mixing. Farinograms showing the effect of detergent are pictured in Fig. 1. It

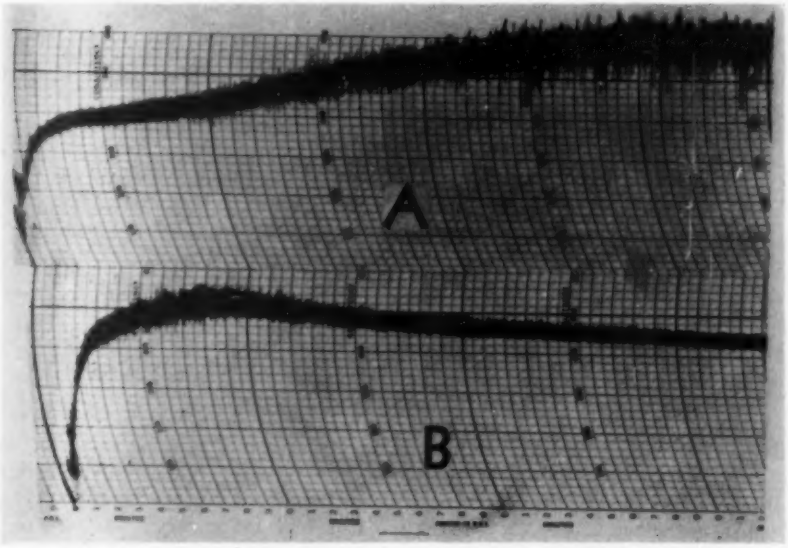

Fig. 1. Farinograms showing the effect of detergent on mixing characteristics of Lee flour. A, 50 g. Lee flour, 35 ml. water, and 0.5 g. Duponol "R." B, 50 g. Lee flour, 35 ml. water.

is apparent that the control dough had broken down well before 35 minutes, while the dough containing detergent maintained nearly maximum resistance to mixing. However, no significant difference in sulfhydryl content of the two doughs was found; values were 0.44 μeq. per g. with detergent and 0.47 without.

General Discussion

The reactivity of the sulfhydryl groups in flour seems to cover a wide range. Some sulfhydryl groups appear to be quite labile, as judged by the substantial loss on mixing in air and by the effect of temperature of the titration mixture, reported earlier. On the other hand, the slowness with which some sulfhydryls are blocked by NEMI and the presence of sulfhydryls in titration slurries after long mixing in air show that some are relatively unreactive or inaccessible. The

differences in reactivity do not appear to be confined either to gluten or nongluten proteins, although those in the soluble proteins have been reported to react more rapidly (9).

The comparisons of sulfhydryl-blocking reagents reported here were made under pH conditions more favorable to reaction than are present in normal doughs. Nevertheless, the greater effectiveness of NEMI, as compared to IAC, in blocking sulfhydryl groups parallels the more marked effect of NEMI on mixing curves (10).

Changes in sulfhydryl content caused by potassium bromate were noticeable only with the fifth-break flour. This flour gives a marked bromate response in baking, and its high protein and sulfhydryl content also provide more favorable circumstances for accurate analyses than are found in the typical flours used in baking. In relating these observations to those of Matsumoto and Hlynka (9), it should be pointed out again that their observations on the effects of bromate and iodate were made on doughs mixed in nitrogen. Our data on fifth-break flour were obtained from doughs mixed in air, so that the decrease in sulfhydryl may be attributed to a bromate effect beyond that caused by air oxidation.[3] The decreases observed are so small, however, that their significance with respect to dough improvement cannot be evaluated without further investigation.

Acknowledgment

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[3] Cunningham and Hlynka (6) have shown that oxygen appears to reduce the rate of reaction of bromate in dough. In doughs made from straight-grade and patent flours mixed in air, the small number of sulfhydryl groups that are reactive to bromate may have been decreased further by air oxidation.

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THE INFLUENCE OF SOYA FLOUR IN BREAD DOUGHS

IV. Alpha-Amylase of Soya[1]

E. MITCHELL LEARMONTH[2] and J. C. WOOD[2]

ABSTRACT

The existence in raw soya of a starch-liquefying enzyme as demonstrated by the amylograph is confirmed. The enzyme is chiefly, if not wholly, a true alpha-amylase. The activity of soya is only of the order of one-hundredth of that of an ordinary wheat malt and it diminishes rapidly with rising temperature and with falling pH, in contrast with that of wheat malt. These results contradict the findings of some other workers and lead to the conclusion that the alpha-amylase activity of raw soya is not significant in commercial breadmaking.

For many years soya has been recognized as a rich source of beta-amylase, but it has been a matter of controversy whether or not it contained more than traces of alpha-amylase. Orestano (8) reported that soya contains only one amylase – beta-amylase. Newton and Naylor (6) found only traces of alpha-amylase in soya beans, both before and after germination. Although their soya amylase had the ability to re-

[1] Manuscript received August 21, 1958.
[2] British Soya Products Limited, Puckeridge, Herts., England.

duce the viscosity of starch paste, they attributed this to beta-amylase. Teller (13) showed that there were two amylases in soya, but Laufer, Tauber, and Davis (3) found only very slight alpha-amylase activity. The work of Peat, Whelan, and Pirt (10) showed the presence of a small amount of alpha-amylase, but they made no attempt to measure it. Ofelt, Smith, and Mills (7), using the amylograph, claimed that soya could be considered an active source of alpha-amylase when compared with a pure alpha-amylase preparation. Learmonth (4) had earlier attempted to measure the alpha activity of several varieties of raw soya beans by the method of Sandstedt, Kneen, and Blish (11) and Stone's modification (12) of it. The latter method is expected to measure traces of alpha activity in supposedly pure preparations of beta-amylase. In no case did he obtain evidence of more than a dubious trace of alpha activity in soya.

The work described here was undertaken to resolve this discrepancy, especially with reference to the use of raw, enzyme-active soya in commercial breadmaking. In the United Kingdom and in Europe, enzyme-active soya is widely used, as a commercial bread improver, in quantities of approximately 1% of the wheat flour in a dough. Ofelt *et al.* (7) had reported so high an alpha-amylase activity for soya (5% raw soya flour equivalent to 12 SKB units of alpha-amylase) as to lead to the supposition on the one hand that it could be used as an alpha-amylase supplement instead of malt or fungal amylase preparations, or on the other, that it could produce deleterious results in doughs that did not require such a supplement. Although their work was confined to soya supplements at the rate of 5% of the wheat starch, their findings were so much at variance with all earlier work and with general bakery experience in the United Kingdom as to require confirmation or disproof.

Their experiments with the amylograph have therefore been repeated and the activity of the soya has been rigorously compared, by the more exact quantitative methods of Sandstedt, Kneen, and Blish (11) and of Peat, Thomas, and Whelan (9) with that of an ordinary commercial wheat malt whose activity (23.0 SKB units) was measured at the same time. To complete the picture, the response of the soya activity to pH and temperature has also been examined in comparison with the wheat malt, and the effect of soya in breadmaking from this point of view is discussed in detail.

Materials and Methods

The soya used was prepared by grinding whole raw beans to a fineness such that less than 2% was retained by a 60 British Standard

sieve. Defatted soya was prepared by Soxhlet extraction of this flour for 4 hours with petroleum ether (b.p. range 30°–40° C.).

Commercial raw wheat starch and a commercial wheat malt were used.

Measurement of Alpha-Amylase Activity. (1) *Amylograph.* The amylograph experiments were carried out exactly as described by Ofelt *et al.* (7). To demonstrate the thermolability of the starch paste-liquefying factor, the following experiment was done:

One gram soya flour was extracted with 100 ml. water at 30°C. for 60 minutes, with shaking every 5 minutes. The extract was centrifuged at 4,500 r.p.m. for 5 minutes and supernatant decanted off. Forty-five milliliters of supernatant (equivalent to 0.45 g. soya flour) were taken for the amylograph test, the volume of water added to the starch being reduced from 405 to 360 ml. A further 45 ml. of supernatant were heated at 62°C. for 15 minutes and tested in the same way.

(2) *Sandstedt-Kneen-Blish Method (11).* The effect of sodium phosphate/citric acid buffers, pH 5.3 and pH 6.6, in a final concentration of 0.01*M* was investigated, together with the effect of raising the temperature of incubation. The micro method of Kneen, Sandstedt, and Hollenbeck (2) was also investigated, but not very thoroughly, for Stone's (12) modification of that method had not given very satisfactory results in earlier work (4). However, a 1% w/v wheat malt flour extract was diluted 1:100 and compared with a 1% w/v soya flour extract.

(3) *Sodium Starch Glycollate Method.* The method of Peat, Thomas, and Whelan (9) was modified as follows: the beta-amylase solution was prepared by extracting 2 g. soya flour with 20 ml. distilled water for 60 minutes at 35°C., with shaking every 5 minutes. The extract was centrifuged and the supernatant removed. Five milliliters of supernatant plus 7.5 ml. 0.2*M* sodium acetate buffer, pH 4.8, well mixed and kept at 62°C. for 15 minutes, cooled immediately to 20°C. and centrifuged. Supernatant used as source of beta-amylase. There was no alpha-amylase activity in this beta-amylase as measured by the Sodium Starch Glycollate Methods I and II (see below).

The substrate was prepared by mixing 200 mg. sodium starch glycollate (British Drug Houses Ltd., Poole, Dorset, England) with 23 ml. water and 1.5 ml. beta-amylase solution (excess) and 9 ml. sodium phosphate/citric acid buffer, pH 5.3 (prepared according to Ofelt *et al.*, 7) or 9 ml. 0.2*M* sodium acetate buffer, pH 6.6.

The substrate was incubated at whatever temperature the digestion was to be done until the Absorption Value (A.V.) fell to a constant level. The A.V. was measured at 680 $m\mu$ in an EEL Portable Colorim-

eter (Evans Electroselenium Limited, Harlow, Essex, England), using 1 ml. test solution, 4 ml. 0.022% w/v iodine in 0.2% w/v potassium iodide, and 1 drop of 6*N* sulfuric acid, made up to 50 ml. The A. V. usually fell by 3 to 5% of the original value.

Preparation of alpha-amylase extracts: 1 g. of the soya flour or wheat malt flour was extracted for 60 minutes at 30°C. with 100 ml. water, with shaking every 5 minutes. The extract was centrifuged at 4,500 r.p.m. for 5 minutes and the residue discarded. The malt extract was diluted before use and the soya extract used as such.

The digests were prepared thus:

Method I: One milliliter alpha-amylase extract to be tested was mixed with 4 ml. substrate and 6 ml. water; 1 ml. removed immediately for A.V. determination. The remainder of the digest was incubated at 35°C. for 5 hours; then 1 ml. was removed for A.V. determination. The percentage fall in A.V. was taken as a measure of the alpha-amylase activity. A.V. was measured as described above, but the final volume was 30 ml. instead of 50 ml.

Method II: Five milliliters test solution with 4 ml. substrate and 2 ml. water were incubated for 60 minutes at the required temperature; A.V. measured immediately before and after incubation as in Method I.

Results and Discussion

The Amylograph. The liquefying effect of unheated soya flour on starch paste, as shown by Ofelt *et al.* (7) with the amylograph, is qualitatively supported by the graphs in Fig. 1. The thermolability of the starch-liquefying factor is also shown. Ofelt *et al.* used soya at the rate of 5% of the wheat starch, a much larger proportion than is customarily used in breadmaking in the United Kingdom. Figure 2 shows the effect of 0.9% addition of enzyme-active full-fat soya – the product customarily used as a bread ingredient in approximately the concentration at which it is used. The liquefying effect is appreciably reduced at this concentration. Figure 2 also shows that the factor is extractable with water and is destroyed by heating the extract for 15 minutes at 62°C. (Fig. 2, b and c). Commercial debittered soya flour was without liquefying power (Fig. 2, d, and Fig. 1, d and e).

It is by no means certain that this starch-liquefying activity of soya is attributable entirely to alpha-amylase. The Z-enzyme of Peat, Thomas, and Whelan (9) may also have some liquefying effect. Z-enzyme would not be measured by the starch glycollate method, however (see below), and its effect, if any, on the amylograph results is considered negligible.

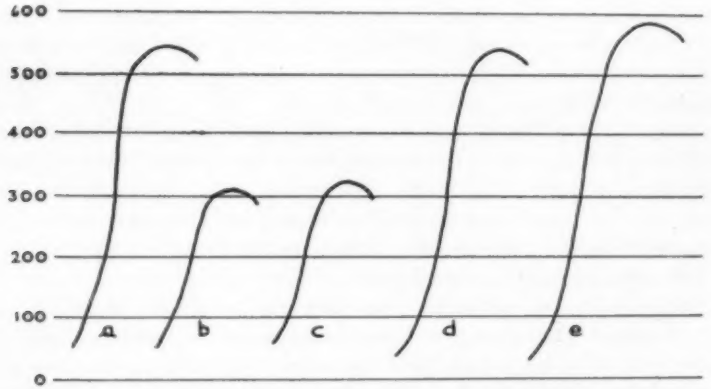

Fig. 1. Amylograph results: a, wheat starch; b, wheat starch + 3.1 g. (6.2%) raw soya flour (assuming 20% fat equivalent to 2.5 g. (5.0%) defatted raw soya flour); c, wheat starch + 2.5 g. (5.0%) defatted soya flour; d, wheat starch + 3.1 g. (6.2%) full-fat soya flour (heat-treated); e, wheat start + 2.5 g. (5.0%) defatted soya flour (heat-treated).

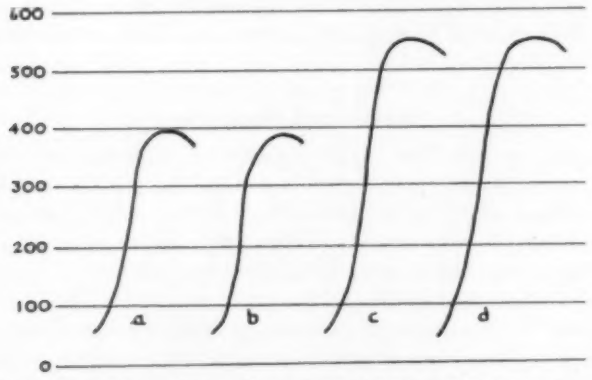

Fig. 2. Amylograph results: a, wheat starch + 0.45 g. (0.9%) raw soya flour; b, wheat starch + 45 ml. 1% aqueous soya flour extract; c, wheat starch + 0.45 ml. 1% aqueous soya flour extract (heated at 62°C. for 15 minutes); d, wheat starch + 0.45 g. (0.9%) soya flour (heat-treated).

Ofelt *et al.* (7) assessed the supposed alpha-amylase activity of their soya flours by comparison with a purified alpha-amylase preparation of known activity, and on this basis arrived at a figure of approximately 12 SKB units for the activity of 2.5 g. of their defatted soya. The evidence for the "known activity" of the purified alpha-amylase does not appear in their paper.

In the United Kingdom the customary source of alpha-amylase for

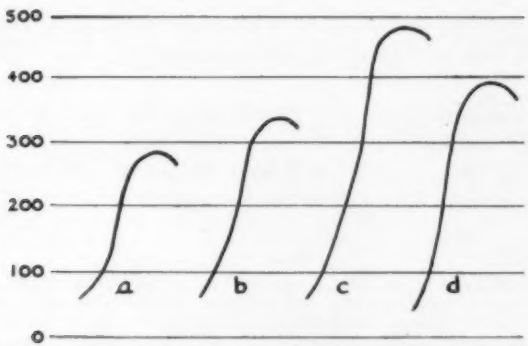

Fig. 3. Amylograph results: a, wheat starch + 20 mg. wheat malt flour; b, wheat starch + 10 mg. wheat malt flour; c, wheat starch + 5 mg. wheat malt flour; d, wheat starch + 450 mg. soya flour.

breadmaking is malt flour, or an active malt extract. Comparison was therefore made of the liquefying power of soya with that of a wheat malt of known Lintner value, and the alpha-activity of the malt flour was also determined by the SKB method. The amylograms are shown in Fig. 3, from which it is clear that 450 mg. of soya were equivalent to somewhere between 5 and 10 mg. of malt flour. Evidently the soya had a liquefying power about one-sixtieth of that of the malt flour. In bakery terms, 2.5 lb. of soya per sack (280 lb.) of wheat flour had the same effect as about 0.75 oz. of malt flour per sack. So small an addition of malt would be ridiculous in bakery and milling practice.

Iodine Staining Methods. (1) *Sandstedt-Kneen-Blish Method (11).* The influence of pH on the alpha-amylase activity of the malt flour, as measured by the method of Sandstedt, Kneen, and Blish (11), is brought out in Table I. It is clear that the alpha-activity of this particular malt (23.0 SKB units) was not exceptionally high, though it had a Lintner value of 83°. Sandstedt, Kneen, and Blish (11) quote figures for the alpha-activity of malts ranging from 39.0 to 17.5 in SKB units. That the alpha-activity of soya, as indicated by the amylograph, was so much

TABLE I

ALPHA-AMYLASE ACTIVITY OF A SAMPLE OF WHEAT MALT FLOUR AND ITS VARIATION WITH TYPE OF BUFFER, pH, AND TEMPERATURE (SKB METHOD)

BUFFER	pH	ACTIVITY	TEMPERATURE
			°C
Sodium acetate/acetic acid	4.6	23.0	30
Sodium acetate/acetic acid	6.6	12.6	30
Sodium phosphate/citric acid	5.3	22.0	30
Sodium phosphate/citric acid	6.6	12.0	30
Sodium phosphate/citric acid	5.3	36.9	45

below this not-very-active malt is a further indication of the negligible influence of soya as a source of alpha-amylase (or starch-liquefying enzyme).

In fact, the activity of soya proved to be too small to be measured by the SKB method, as had been found earlier (4), and attention turned to the micro method of Kneen, Sandstedt, and Hollenbeck (2). The results obtained by this method are given in Table II. Sandstedt

TABLE II

ALPHA-AMYLASE ACTIVITY (KNEEN, SANDSTEDT, and HOLLENBECK MICRO METHOD) OF SOYA AND WHEAT MALT IN DIFFERENT BUFFERS. TEMPERATURE 30°C THROUGHOUT

SOURCE	BUFFER	pH	ALPHA-AMYLASE UNITS
1% w/v Aqueous soya flour extract	Sodium acetate/acetic acid	4.6	0.081
1% w/v Aqueous soya flour extract	Sodium phosphate/citric acid	5.3	0.115
1% w/v Aqueous soya flour extract	Sodium phosphate/citric acid	6.6	0.148
1% w/v Aqueous wheat malt flour extract diluted 1:100	Sodium acetate/acetic acid	4.6	0.100

has already pointed out that the unit of measurement obtained by the micro method is not the same as the SKB unit of the earlier paper. In terms of the micro-method units, it is clear from Table II that the soya has only one one-hundred-twenty-fifth of the activity of the malt at pH 4.6.

An interesting contrast appears in the influence of pH on the alpha-activity of soya and of malt. That of soya increases by nearly 50% as the pH rises from 4.6 to 6.6 (Table II), whereas that of malt falls about the same amount over the same pH range (Table I). This comparison is fairly drawn from the figures obtained, since both gross and micro methods measure the same end point in the same reaction. Hollenbeck and Blish (1) observed a similar trend with wheat malt.

(2) Sodium Starch Glycollate Method. The most convenient method of studying alpha-amylase proved to be the Sodium Starch Glycollate Method of Peat, Thomas, and Whelan (9), modified as described above. Though the method is open to criticism on the ground that the substrate is an artifact foreign to bread dough, it offers a convenient means of comparing the alpha-activities of similar sources of the enzyme and is especially useful for measuring the very low activities found in soya.

Table III compares the activities of seven different samples of raw soya beans in terms of the percentage fall in Absorption Value of the digest after incubation, as described in the first modification (Method I; see "Materials and Methods" section) of the method developed by

TABLE III

ALPHA-AMYLASE ACTIVITY OF SOYA BEANS OF DIFFERENT GEOGRAPHICAL ORIGIN (SODIUM STARCH GLYCOLLATE–METHOD I)

SAMPLE	PERCENT FALL: ABSORPTION VALUE	SAMPLE	PERCENT FALL: ABSORPTION VALUE
	%		%
A	22.6	E	11.3
B	17.7	F	9.7
C	17.7	G	4.8
D	14.5	H	4.8

Peat, Thomas, and Whelan (9). Sample A was the material used in the amylograph tests and in the micro method of Kneen, Sandstedt, and Hollenbeck (2) (see above). It was clearly the most active of the seven, and the other samples would have had even less effect on the viscosity of starch. All were commercial samples of different geographical origin. The fivefold variation in activity is of no significance in breadmaking, since the most active sample has so small a potency in comparison with malt.

The activity of the wheat malt as measured by this method is shown in Fig. 4, where the dilution of a 1% extract is plotted against the percentage fall in absorption value. The fall in pH from 6.6 to 5.3 is accompanied by a *rise* in alpha-amylase activity of 33%. The effect of an identical pH change on the alpha-activity as measured by SKB method can be seen (Table I) to be an increase of 45%. With soya, on the other hand (Table IV), a fall in pH from 6.6 to 5.3 (the range over

TABLE IV

SOYA FLOUR ALPHA-AMYLASE ACTIVITY. EFFECT OF CHANGE OF BUFFER AND PH (SODIUM STARCH GLYCOLLATE–METHOD I. TEMPERATURE 35°C. THROUGHOUT)

BUFFER	PH	PERCENT FALL: ABSORPTION VALUE
		%
Sodium acetate/acetic acid	6.6	30.5
Sodium phosphate/citric acid	5.3	21.0
Sodium phosphate/citric acid	6.6	31.2

which the pH of a bread dough is likely to fall from the time of mixing to the beginning of baking) is accompanied by a *fall* in alpha-activity of 31%, as indicated by the Glycollate Method, compared with a fall of 22% over the same pH range in the micro method of Kneen, Sandstedt, and Hollenbeck (2) (Table II). The discrepancy between the two methods in this response to pH is not of much significance in view of the very low activities of all soya varieties tested. It may be due to inherent inaccuracy in the micro method.

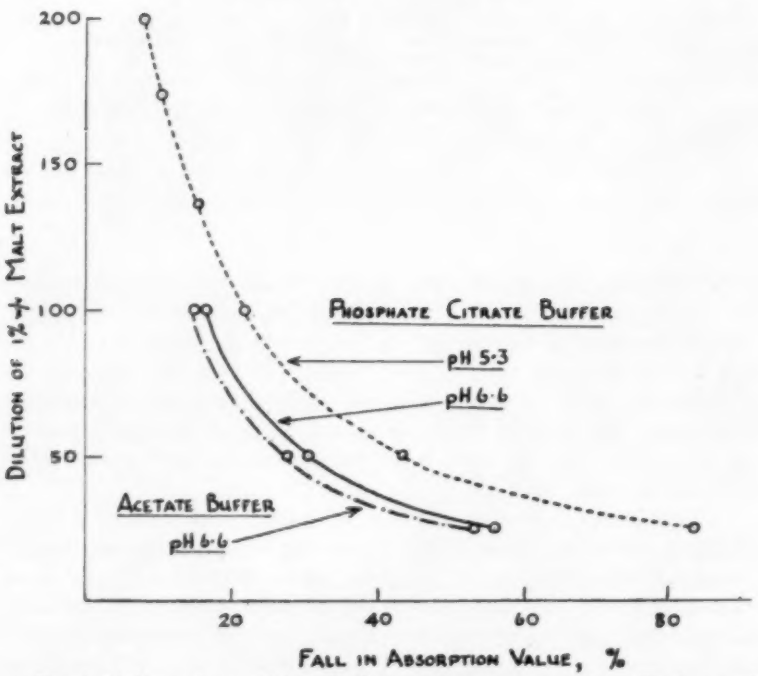

Fig. 4. Alpha-amylase activity of wheat malt extract at various dilutions and different pH values (Sodium Starch Glycollate–Method I).

The most active soya sample, A, giving a fall in absorption value of 22.6%, has an activity only one-ninetieth of that of the malt, and the other samples range downwards to negligible values. This method, therefore, gives results in general agreement with those of the micro method of Kneen, Sandstedt, and Hollenbeck and with the amylograph, so far as this can be used for quantitative assessment.

Effect of Temperature. Sodium Starch Glycollate Method was used to study the response to temperature changes of the alpha-amylase from both soya and malt. The long incubation time adopted in the first modification of the original method gave anomalously low results for malt as the temperature rose, and it seemed probable that some loss of activity was occurring during the incubation owing, perhaps, to digestion by the proteolytic enzymes in malt. The second modification (Method II; see "Materials and Methods" section) overcame this difficulty and enabled comparable measurements at five different temperatures to be made of the malt extract at five different dilutions and of the soya (sample A) at a single concentration (1%). These results

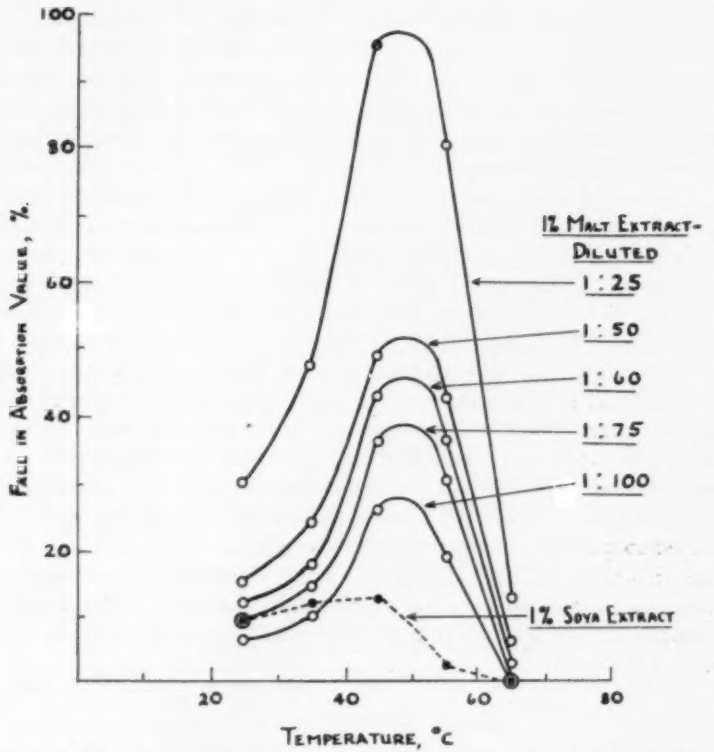

Fig. 5. Effect of temperature on wheat malt flour and soya flour alpha-amylase activities (Sodium Starch Glycollate–Method II).

are grouped in Fig. 5.

The soya alpha-amylase showed little increase with rise in temperature from 25° to 45°C., and beyond this point it fell rapidly to a scarcely measurable activity at 55°C. and complete extinction at 65°C. The 1% malt extract, on the other hand, even when diluted 100 times, showed a sharp increase in activity with rise in temperature, having a peak about 50°C. Thereafter the activity declined, but at 55°C. was still greater than that of soya at any temperature. More concentrated extracts of malt had proportionately greater activity. They also had markedly greater resistance to temperature; the 1:25 dilution of 1% malt extract at 65°C. was still as active as the 1% soya extract at its peak value.

This variation in resistance to temperature with concentration raises interesting questions about the true thermal death point of en-

zymes in the dough during baking. It is difficult to assess the true water-enzyme ratio in a dough, for some of the added water may not be "available." The "availability" of water in a bread dough is a very uncertain matter and becomes more obscure when baking begins. It is therefore difficult to interpret these data in terms of fermentation or baking behavior of doughs.

It is well known that the stability at high temperatures of most, if not all, enzymes is greatly increased as available moisture is reduced. Clearly the stability of alpha-activity of a malt supplement added to a dough at the rate of 0.5 to 1% of the wheat flour (and therefore of the order of 1 to 2% of the total water present) is likely to be appreciably higher than that found for the dilute extracts used in these experiments. The gap between the minute activity of soya and the useful value of malt at fermentation temperatures can only widen as the temperature rises. It is abundantly clear, however, that soya, having a negligible alpha-amylase activity at fermentation temperature, can have little or no effect on starch breakdown from this cause at this stage; and since its activity rises so little with temperature, and is destroyed so quickly at temperatures above 45°C., it cannot, in the quantities in which it is used (about 1% of the wheat flour) contribute anything appreciable to the total alpha-amylase activity of the dough in comparison with malt, or indeed, the wheat flour itself when the bread goes to the oven.

Conclusion

The existence of a starch-liquefying enzyme in raw soya has been confirmed and it has been shown, even in the most potent sample, to be quite negligible in comparison with a sample of malt of normal activity. It is clear that the small liquefying effect of soya is attributable chiefly, if not wholly, to a true alpha-amylase, since Z-enzyme is not measured by the Sodium Starch Glycollate Method, and the ratio of soya activity to malt activity is of the same order when measured by both methods.

The small activity of soya diminishes as pH falls during fermentation and vanishes rapidly as temperature rises. In sum, it cannot have a significant effect on starch breakdown during either fermentation or baking of bread.

The use of soya in breadmaking is not likely, therefore, to contribute on the one hand to difficulties associated with excessive alpha-amylase activity, for example in sprouted wheats, or, on the other hand, to the useful alpha-amylase of malt or fungal enzyme supplements where these are required. In view of its antiproteolytic activity

(4) when prepared from selected beans (5), it does offer a useful natural means of controlling the undesirable proteolysis sometimes produced by malt.

Acknowledgments

The gift of a sample of pure beta-amylase from Stanley Peat is gratefully acknowledged, as are helpful discussions with E. J. Bourne, W. J. Whelan, Gwen J. Walker, and J. R. Turvey.

Thanks are also due to the Board of British Soya Products Limited for permission to publish this paper.

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EFFECT OF FREEZING, DEFROSTING, AND STORAGE CONDITIONS ON THE FRESHNESS OF DINNER ROLLS AND CINNAMON ROLLS[1]

K. KULP[2] AND W. G. BECHTEL[2]

ABSTRACT

Freshly baked dinner rolls and cinnamon rolls wrapped in cellophane were frozen at 0°F. (−18°C.) without forced air and at −20°F. (−29°C.) with forced air at 1,000 linear ft. per minute. Defrosting was conducted at 75°F. (24°C.) without forced air and at 115°F. (46°C.) with forced air at 1,000 linear ft. per minute. Freezing and defrosting caused a slight decrease in freshness and increase in firmness, but not as much as when the rolls were stored at 70°F. (21°C.) for 24 hours. Only small differences in freshness and firmness were caused by the variations of the above freezing and defrosting operations. Unwrapped rolls can be frozen at 0°F. (−18°C.) or −20°F. (−29°C.) at air velocities from 0 to 1,000 ft. per minute without serious reduction of moisture. It is undesirable to defrost rolls unwrapped, in view of an appreciable moisture loss. Wrapped dinner rolls and cinnamon rolls can be stored at 0°F. (−18°C.) and at −20°F. (−29°C.) for at least 2 months without critical decrease of freshness. Rapid firming and loss of freshness occurred during storage at 10°F. (−12°C.) or above.

Pence and co-workers have made a thorough study of the effect of wide variations in conditions of freezing, frozen storage, and defrosting on the freshness and firmness of bread (12,14,15). Recently they have reported similar studies on cakes (11,13). While there is great interest in the freezing of sweet yeast-raised products, only a few studies have appeared in the literature, and these have been limited in scope. Arnold (2) reported that the quality of rolls frozen and stored at 0°F. (−18°C.) was satisfactory. Gordon (8) found that sweet yeast-raised products frozen at 8° to 12°F. (−13° to −11°C.) and stored unwrapped were salable for 5 to 6 days. Charles and Van Duyne (7) and Nenninger (10) studied the quality of rolls frozen and stored at −10°F. (−23°C.), while Beattie *et al.* (3) found that coffeecake and butterfly buns frozen at −25°F. (−32°C.) and stored at −15°F. (−26°C.) were satisfactory after 9 months.

It was the purpose of this investigation to study systematically the effect of different conditions of freezing, frozen storage, and defrosting on the freshness and firmness of soft dinner rolls and a typical

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[2] American Institute of Baking, Chicago, Illinois.

sweet yeast-raised product, cinnamon rolls.

Materials and Methods

Dinner Rolls and Cinnamon Rolls. Rolls were prepared in the experimental bakery of the American Institute of Baking. The formulas (see Table I) were selected to produce products intermediate between the lean and rich of commercial practice. Dinner rolls were baked in a pan 7 by 7 by 1½ in., nine rolls per pan. This unit weighed 8 oz. Cinnamon rolls were baked in a pan 8 by 8 by 1¾ in., 12 rolls per pan. The unit weighed 16 oz. Moisture content of the fresh rolls, determined by the vacuum-oven procedure (1), was 33% for dinner rolls and 26% for cinnamon rolls.

TABLE I
FORMULAS OF DINNER ROLLS AND CINNAMON ROLLS

	Dinner Rolls[a]	Cinnamon Rolls[a]
	%	%
Sponge		
Flour	60	76
Water	36	52
Yeast	3	8
Dough		
Flour	40	24
Water	29	0
Salt	2	2.5
Sugar	10	16
Nonfat dry milk	6	6
Eggs	0	16
Shortening	10	16
Raisins	0	20
Cinnamon	0	0.5

[a] Total flour is 100%. Other ingredients are given as percentages of the total flour.

Freezing and Defrosting. Units of the freshly baked products equilibrated at 70°F. (21°C.) were placed in a freezer equipped with variable thermoregulator which maintained a temperature within ± 2°F. (± 1.1°C.) of the setting. Air circulation at rates up to 1,000 linear ft. per minute across the products was provided by an electric blower. This equipment has been described in more detail (9). The time-temperature relationships for freezing and defrosting were also given.

Packaging. Both kinds of rolls were packaged in 300 MST (moisture proof, heat-sealing, transparent) cellophane and the package was sealed.

Moisture Loss on Freezing and Defrosting. The fresh rolls were cooled to room temperature and weighed. For moisture loss on freezing the rolls were frozen unwrapped. At the end of the freezing time they were wrapped and sealed in waxed paper to prevent further change of

moisture, after which they were defrosted, unwrapped, and weighed. For moisture loss on defrosting, the rolls were cooled and weighed. Then they were wrapped and sealed in waxed paper, frozen, and equilibrated at 0°F. (−18°C.). The wrapper was removed just prior to defrosting, they were then weighed, defrosted to 70°F. (21°C.), and again weighed. Loss of weight was taken as the moisture loss.

Relative Humidity. This was determined by means of a wet- and dry-bulb thermometer in a current of air provided by a small electric fan.

Firmness. The Baker Compressimeter (1) was used. Slices of center crumb 1 by 1 by 1½ in., cut in a miter box, were compressed 2.5 mm. Each value is the average of tests of 20 slices. Freshly baked products were tested 2 hours after removal from the oven.

Freshness. A sensory panel of 20 members was used. Panel members were selected by the method described by Bechtel and Meisner (4), to make sure that they were able to detect the changes generally associated with staling. A symmetrical 8-point rating scale was used, with ratings *very fresh, fresh, moderately fresh, slightly fresh, slightly stale, moderately stale, stale,* and *very stale.* For statistical analysis the rating *very stale* was given the value 1. Successive ratings were given consecutive integers to 8 for *very fresh.* Each result is the average of two panel tests.

Results and Discussion

Effect of Freezing and Defrosting Rates on Firmness and Freshness. The relationship of temperature, air flow, and packaging to the rates of freezing and defrosting of dinner rolls and cinnamon rolls has been reported (9). To study the effect of rates of freezing and defrosting on freshness and firmness the rolls were frozen and defrosted, packaged, at the slowest and most rapid rates previously employed. Freezing conditions were: (a) freezer at 0°F. (−18°C.) without forced air, and (b) −20°F. (−29°C.) with an air flow of 1,000 linear ft. per minute. Defrosting conditions were: (a) defrosting cabinet at 75°F. (24°C.) without forced air, and (b) 115°F. (46°C.) with an air flow of 1,000 linear ft. per minute. The cooling time, the time required to lower the temperature at the center of the unit from 70°F. (21°C.) to 10°F. (−12°C.) was used as an index of the rate of freezing. Defrosting time was that required to raise the temperature at the center of the unit from 0°F. (−18°C.) to 70°F. (21°C.).

The freezing and defrosting times under the above conditions are given in columns 1 and 2 of Table II.

Rolls were defrosted after 20 hours in the freezer, so that very little,

TABLE II

EFFECT OF FREEZING AND DEFROSTING RATES ON THE FRESHNESS AND FIRMNESS OF DINNER ROLLS AND CINNAMON ROLLS

No.	Cooling Time[a]	Defrosting Time[b]	Freshness Rating[c]	S.D.[d]	Firmness Value	S.D.[d]
	minutes	*minutes*			*g/sq in*	
			Dinner Rolls			
1	Freshly baked[e]		7.30	0.80	13.3	1.34
2	55	40	6.55	0.92	13.5	1.20
3	55	145	6.55	1.00	13.9	2.49
4	163	40	6.58	1.34	13.3	2.54
5	163	145	6.68	0.92	16.0	3.78
6	After 24 hours at 70°F. (21°C.)		6.03	1.28	19.0	3.28
			Cinnamon Rolls			
1	Freshly baked[e]		6.90	0.98	17.7	3.20
2	55	27	6.28	1.30	24.3	4.92
3	55	157	6.03	1.33	22.0	7.00
4	130	27	6.10	1.25	22.1	4.85
5	130	157	5.63	1.22	23.4	3.50
6	After 24 hours at 70°F. (21°C.)		4.95	1.33	34.3	5.83

[a] From 70°F. (21°C.) to 10°F. (−12°C.).
[b] From 0°F. (−18°C.) to 70°F. (21°C.).
[c] Rating scale: Very fresh 8, fresh 7, moderately fresh 6, slightly fresh 5, slightly stale 4, moderately stale 3, stale 2, very stale 1.
[d] Standard deviation.
[e] Two hours after baking.

TABLE III

ANALYSIS OF VARIANCE APPLIED TO THE EXPERIMENTS IN TABLE II

		Dinner Rolls		Cinnamon Rolls
Freshness ratings				
Source of variation	D.F.		M.S.	M.S.
Total	119		1.0	1.6
Judges	19		2.3**	3.9**
Treatment	5		3.1**	8.6**
(a) fresh vs. others[a]		1	10.9**	20.4**
(b) 24 hours vs. frozen		1	4.3**	17.8**
(c) slowest vs. other rates		1	0.2 N.S.	4.4*
(d) among fast rates		2		0.3 N.S.
Treatment × Judges	95		0.6	0.8
Firmness values				
Total	119		11.4	59.9
Treatment	5		114.3**	615.3**
(a) fresh vs. others		1	56.4**	942.5**
(b) 24 hours vs. frozen		1	372.5**	2,061.2**
(c) slowest rates vs. others		1	88.8**	24.3 N.S.
(d) among fast-frozen		2	1.9 N.S.	
Residual (error)	114		7.4	35.5

[a] a, No. 1 vs. 2-6 of Table II; b, No. 6 vs. 2-5 of Table II; c, No. 5 vs. 2-4 of Table II; d, Among No. 2-4 of Table II.

if any, of the changes in freshness and firmness can be attributed to time of storage. The processes of freezing and defrosting caused some

loss of freshness and increase in firmness, but the changes were much less than in rolls stored at 70°F. (21°C.) for 24 hours.

As shown in Table II, freshly baked dinner rolls were given a freshness rating between *fresh* and *very fresh* (7.30). After freezing and defrosting this was lowered to between *fresh* and *moderately fresh* (6.55 to 6.68), while after 24 hours at 70°F. (21°C.) the rating was *moderately fresh* (6.03). As shown in Table III, freshly baked dinner rolls were significantly fresher than the others, and the frozen rolls were significantly fresher than those stored 24 hours at 70°F. (21°C.). Within the experimental limits employed, differences in freezing and defrosting times had little effect on freshness as measured by the sensory panel.

Except when both freezing and defrosting were slow, these processes had only a slight effect on firmness of dinner rolls. Slow freezing and defrosting caused a significant increase in firmness over the procedures where at least one of the operations was conducted at a rapid rate. Those stored for 24 hours at 70°F. (21°C.) were significantly firmer than those which had been frozen.

Results of tests of cinnamon rolls in Table II show a somewhat similar relationship. Freezing and defrosting caused a loss of freshness, but that was significantly less (Table III) than that caused by storage for 24 hours at 70°F. (21°C.). Rate of freezing and defrosting had a greater effect on freshness than in the case of dinner rolls. When cinnamon rolls were frozen and defrosted at the slowest rates there was a significant loss of freshness beyond that caused by any of the other combinations of freezing and defrosting rates. The process of freezing and defrosting caused a material increase in firmness, but there was no significant difference in firmness due to the changes in rate of freezing and defrosting. Storage for 24 hours at 70°F. (21°C.) caused a significantly greater increase in firmness than did freezing and defrosting.

Freezing and defrosting caused approximately half the loss of freshness that was caused by 24-hour storage at 70°F. (21°C.); this indicates that there is an advantage to freezing rolls unless they are consumed on the day they are baked. The data show, also, that it is desirable to freeze and defrost these products rapidly to minimize loss of freshness and softness.

Moisture Loss on Freezing and Defrosting Unwrapped Rolls. During freezing, unwrapped rolls were exposed to the atmosphere of the freezer for the time required for the temperature at the center of the unit to fall from 70°F. (21°C.) to a temperature 10°F. (5.6°C.) above that of the freezer. Freezer temperatures of 0°F. (−18°C.) and −20°F. (−29°C.) were employed, with air flow of 0, 150, 500, and 1,000 linear ft. per minute. The exposure times of dinner rolls were from 68 min-

utes to 162 minutes, depending on temperature and air flow rate. Those of cinnamon rolls were from 48 minutes to 173 minutes.

No relationship was found between moisture loss and freezer temperature or rate of air flow. The 8-oz. unit of dinner rolls lost an average of 0.5% of its initial weight, with a range of 0.2 to 0.8%. The 16-oz. unit of cinnamon rolls lost an average of 0.3% of its initial weight, with a range of 0.1 to 0.4%.

On defrosting, the unwrapped rolls were exposed for the time required for the temperature at the center of the unit to rise from 0°F. (−18°C.) to 70°F. (21°C.). Defroster temperatures of 75°F. (24°C.), 95°F. (35°C.), and 115°F. (46°C.) were used and at each temperature air flow rates were 0, 150, 500, and 1,000 ft. per minute. The average moisture loss from dinner rolls was 1.4%, with a range from 0.6 to 2.44%. The average moisture loss from cinnamon rolls was 1.0%, with a range from 0.6 to 1.6%. Analysis of variance showed no significant relationship between moisture loss and either defroster temperature or rate of air flow.

During freezing the surface layers of the product are cooled rapidly and the rate of evaporation of moisture is diminished because of the lowered moisture vapor pressure. On defrosting the reverse is true, and as the product is warmed evaporation of moisture becomes greater. The moisture losses on freezing were not high, and it appears feasible to freeze rolls prior to wrapping if this is desirable from other points of view. The combined moisture loss due to freezing and defrosting averaged 1.9% for dinner rolls and 1.3% for cinnamon rolls. These were the result of minimum exposure to the atmosphere of the freezer and defroster and do not include losses which would occur during frozen storage, nor those which would occur if the rolls were allowed to remain longer than the minimum time in the defroster. It has been shown (5) that a loss of 2% moisture from bread results in significant lowering of its freshness. It seems probable that such a moisture loss would affect the freshness of rolls in a similar manner. It therefore appears undesirable to defrost rolls unwrapped.

Effect of Storage Temperature on Freshness and Firmness. The importance of freezer temperature to the maintenance of freshness and softness of dinner rolls is shown in Table IV. The rolls were frozen in 55 minutes and defrosted in 145 minutes. It was shown in Table II that rolls treated in this manner were given the freshness rating 6.55 and had a firmness of 13.9 g. per sq. in. During the first week of storage there was a decrease in freshness and an increase in firmness at all temperatures. Except for the rolls stored at 20°F. (−7°C.), the loss of freshness was not great. At 20°F. (−7°C.) the freshness rating after 1

week was 4.85, a value below *slightly fresh*. This was approximately the same as that of rolls stored at 10°F. (−12°C.) for 3 weeks. At these two higher temperatures there was a substantial increase in firmness within 1 week and a further increase on longer storage.

TABLE IV

EFFECT OF STORAGE TIME AND TEMPERATURE ON THE FRESHNESS AND FIRMNESS OF DINNER ROLLS[a]

Storage Temperature		Time of Frozen Storage					
		1 Week		2 Weeks		3 Weeks	
		Freshness Rating[b]	S.D.[c]	Freshness Rating[b]	S.D.[c]	Freshness Rating[b]	S.D.[c]
°F	°C						
20	(− 7)	4.85	1.24	4.25	1.13	4.15	1.49
10	(−12)	6.28	0.89	5.98	1.97	4.60	1.57
0	(−18)	6.05	1.29	5.95	1.05	6.08	1.05
−20	(−29)	5.93	1.40	6.20	1.13	5.78	1.24
		Firmness					
		g/sq in	*S.D.*	*g/sq in*	*S.D.*	*g/sq in*	*S.D.*
20	(− 7)	32.8	5.94	31.2	3.04	39.8	3.02
10	(−12)	29.2	3.43	33.9	5.99	46.7	6.12
0	(−18)	28.5	3.15	20.7	2.47	22.1	2.62
−20	(−29)	21.9	5.93	21.7	4.36	17.8	3.09

[a] Values 2 hours after baking: freshness, 7.00 (S.D. 0.75); firmness 15.8 g. per sq. in. (S.D. 2.42).
[b] Based on the rating scale: 8, very fresh; 7, fresh; 6, moderately fresh; 5, slightly fresh; 4, slightly stale; 3, moderately stale; 2, stale; 1, very stale.
[c] Standard deviation.

At freezer temperatures of 0°F. (−18°C.) or below, there was no evidence of staling or firming from the first to third week of storage. The study at 0°F. (−18°C.) was continued for a total storage time of 8 weeks. The freshness rating after the eighth week was 6.35 and the firmness was 21.9 g. per sq. in., showing that no further deterioration in quality had occurred.

As shown in Table V, freshness and firmness values at 20°F. (−7°C.) were significantly below those at lower temperatures, while the values at 10°F. (−12°C.) were significantly below those at 0°F. (−18°C.). If dinner rolls are to be frozen and stored for more than a few days a temperature of 0°F. (−18°C.) or below is required to maintain freshness and softness.

Cinnamon rolls were frozen in 55 minutes and defrosted in 157 minutes. This treatment reduced the freshness to 6.03 or *moderately fresh*, and increased the firmness to 22.0 g. per sq. in. (Table II). Data in Table V show that after storage for 1 week at 20°F. (−7°C.) the rolls were rated 4.28, or *slightly stale*, and the firmness had increased to 40.0 g. per sq. in. These values are appreciably poorer than those in Table

II for cinnamon rolls stored 24 hours at 70°F. (21°C.). A further loss of freshness and increase in firmness occurred during the following 2 weeks.

The deterioration of cinnamon rolls at 10°F. (−12°C.) was rather rapid. At the end of the first week they were rated 5.13 in freshness *(slightly fresh)*. Firmness had increased to 33.2 g. per sq. in. These values are approximately the same as for rolls stored at 70°F. (21°C.) for 24 hours. During the next two weeks freshness continued to decrease to a value not appreciably better than that of the rolls at 20°F. (−7°C.).

TABLE V

EFFECT OF STORAGE TIME AND TEMPERATURE ON THE FRESHNESS AND FIRMNESS OF CINNAMON ROLLS[a]

Storage Temperature		Time of Frozen Storage					
		1 Week		2 Weeks		3 Weeks	
		Freshness Rating[b]	S.D.[c]	Freshness Rating[b]	S.D.[c]	Freshness Rating[b]	S.D.[c]
°F	°C						
20	(− 7)	4.28	1.07	3.60	1.58	3.75	1.40
10	(−12)	5.13	1.23	4.83	1.45	3.88	1.38
0	(−18)	6.45	1.22	5.90	1.02	5.55	1.65
−20	(−29)	5.90	1.60	5.70	1.30	5.60	0.99
		Firmness					
		g/sq in	*S.D.*	*g/sq in*	*S.D.*	*g/sq in*	*S.D.*
20	(− 7)	40.0	11.27	48.7	14.96	63.8	18.18
10	(−12)	33.2	5.97	32.4	7.74	44.5	8.16
0	(−18)	15.8	4.17	18.1	5.09	20.8	5.08
−20	(−29)	14.6	5.20	16.6	3.75	15.4	3.40

[a] Values 2 hours after baking: freshness 6.90 (S.D. 0.96); firmness, 14.2 g. per sq. in. (S.D. 2.86).
[b] Based on the rating scale: 8, very fresh; 7, fresh; 6, moderately fresh; 5, slightly fresh; 4, slightly stale; 3, moderately stale; 2, stale; 1, very stale.
[c] Standard deviation.

The freshness rating of cinnamon rolls after 1 week at 0°F. (−18°C.) appears to be abnormally high compared to the value for the frozen and defrosted rolls in Table II. This may reflect some variation in the characteristics of the different lots of rolls, together with the normal day-to-day variation to be expected of the panel. If one uses the rating approximately 6.0, which seems reasonable from Table II and from the value at −20°F. (−29°C.) after 1 week, then cinnamon rolls showed no loss of freshness (beyond that caused by freezing and defrosting) during the first 2 weeks. The small increase in firmness was negligible. There was apparent deterioration in freshness during the third week and the study was continued for a total of 8 weeks to determine if staling would continue. Tests were made at 2-week intervals. A further loss of freshness was observed at each test until, after 8 weeks, the

rolls were rated *slightly stale* (4.05). Since the firmness after 8 weeks was only 23.4 g. per sq. in., it seems unlikely that firming was involved to any great extent in the loss of freshness. During the 3-week storage study at −20°F. (−29°C.) this temperature appeared to offer no advantage over 0°F. (−18°C.) in maintaining freshness, although it prevented any firming.

The analysis of variance in Table VI shows that deterioration at 20°F. (−7°C.) was significantly greater than at the lower temperatures and that the loss of freshness and increase in firmness at 10°F. (−12°C.) were significantly greater than those at 0°F. (−18°C.) and −20°F. (−29°C.). There was no significant difference between the results at the last two temperatures.

TABLE VI

ANALYSIS OF VARIANCE APPLIED TO THE EXPERIMENTS IN TABLES IV AND V

		Dinner Rolls	Cinnamon Rolls
Freshness ratings			
Source of variations	D.F.	M.S.	M.S.
Total	319	2.5	2.5
Storage time (S)	3	104.2 **	79.9 **
Judges (J)	19	3.8 **	4.3 **
Temperature (T)	3	25.0 **	43.2 **
+20° vs. −20°, 0° and 10°F.	1	69.1 **	82.3 **
−20° vs. 0°F.	1	0.1 N.S.	1.2 N.S.
−20° & 0°F. vs. +10°F.	1	5.9 **	46.3**
S × J	57	1.3 N.S.	1.3 N.S.
T × J	57	1.4 N.S.	0.6 N.S.
S × T	9	5.2 **	5.8 **
S × T × J	171	0.9	1.0
Firmness values			
Total	319	130.4	289.9
Storage time	3	3,731.1 **	6,669.9 **
Temperature	3	2,838.7 **	12,393.5 **
+20° vs. −20°, 0° and 10°F.	1	1,978.0 **	25,235.4 **
−20° vs. 0°F.	1	245.0 N.S.	164.0 N.S.
−20° & 0°F. vs. +10°F.	1	6,293.0 **	11,781.0 **
Residual	313	69.9	112.7

Bergholz (6) observed that when raisin bread is stored at room temperature, moisture migrates from the crumb to the raisins. A similar change occurs in cinnamon rolls, even during frozen storage. This is evident from the results in Table VII.

After 3 weeks of storage the moisture gain of raisins was 11.5% at +20°F. (−7°C.), 8.6% at 0°F. (−18°C.), and 3.4% at −20°F. (−29°C.). Part of this moisture change undoubtedly occurred during the freezing and defrosting operations, but at 0°F. (−18°C.) and +20°F. (−7°C.) it was too high to be accounted for by this effect only. It is concluded, therefore, that moisture migration is not arrested entirely in the freezer and is at least partly responsible for the relatively low stability of this

TABLE VII

MOISTURE REDISTRIBUTION IN CINNAMON ROLLS DURING STORAGE

CONDITION OF STORAGE	MOISTURE	
	In Raisins	In Crumb
	%	%
Freshly baked	21.0	25.1
72 hours at 70°F. (21°C.)	30.6	22.0
3 weeks at 20°F. (−7°C.)	32.5	21.1
3 weeks at 0°F. (−18°C.)	29.6	22.1
3 weeks at −20°F. (−29°C.)	24.4	23.0

product. This phenomenon would probably be a factor in the stability during frozen storage of other bakery foods containing raisins, or of bakery foods consisting of more than one phase, one of which differs in hygroscopicity or freezing point from the other.

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PILOT-PLANT STUDIES ON THE CONTINUOUS BATTER PROCESS TO RECOVER GLUTEN FROM WHEAT FLOUR[1]

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ABSTRACT

Certain variables involved in carrying out the continuous batter process for separating gluten and starch from wheat flour, such as water-to-flour ratio, retention time, temperature of mixing water, and mixer speed, were studied to determine their effect on gluten protein recovery and gluten purity. Generally, the recovery was increased by increasing the mixing-water temperature and by thickening the batter. However, the purity of the gluten product increased as the batter became thinner. Processing also appeared to be affected by ash content of the flour. As the ash content increased, less gluten was recovered, and the gluten also contained less protein.

Cost estimates for processing 200,000 lb. of flour daily to millstarch (crude starch slurry) and to wet gluten fractions indicate a plant investment cost of $316,100 and a processing cost of 26.5 cents per 100 lb. of flour processed.

A previous article describes a pilot plant for the separation of gluten and starch from wheat flour by a continuous batter process (2). Its construction and operation were simpler than other continuous or batch processes described in the literature (4,5,6). The versatility of the pilot plant was demonstrated by processing flours milled from various types of wheat. In these experiments, all processing variables were held as constant as possible, except the amount of mixing water. Further investigations were made on the effects of a number of processing variables on the recovery of the flour protein in the gluten, and other work was carried out on the processing of low-grade flours milled from hard wheat that had a wide variety of chemical and physical properties. Results of these investigations and estimates of plant investment and operating costs for carrying out the process are now being reported.

Material and Methods

Commercial, untreated, first and second clear flours milled from hard red wheat were processed in 100-lb. lots, and each continuous run lasted 1 hour. The moisture of each flour was determined by drying a sample for 4 hours at 110°C. under a vacuum of 28 in. of mercury. Protein ($N \times 5.7$) was determined by the improved Kjeldahl method for nitrate-free samples (1). Ash was determined according

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to the official method of analysis of the AOAC (3). Solubles were determined by shaking 1 g. of flour in 100 ml. of distilled water intermittently for 30 minutes, centrifuging, and then analyzing an aliquot of the supernatant for total solids. Analytical determinations were reported on a moisture-free basis (M.F.B.).

Briefly, the continuous process involves mixing 1 part of flour with from 1 to 1.8 parts of water, depending on the flour being used, in a continuous mixer until a smooth, elastic batter is formed. This batter is withdrawn continuously to a pump, where water is added to make the ratio of water to flour about 3 to 1. Starch is washed from the batter leaving the gluten in the form of small curds. Starch and gluten are then separated on a shaker screen. The gluten curds are further washed by passing them through one or more additional pumps with added water, and the gluten is recovered on screens.

Results and Discussion

Effect of Operating Variables on the Processing of a First Clear Flour. The following process variables were investigated, using a first clear flour milled from a hard red wheat: a), water-to-flour ratio, b), holding or aging, c), r.p.m. of the washing or "cutting" pump, and d), size of screen mesh used on the shaker screen. The chemical analysis of the flour (M.F.B.) was: protein, 16.6%; ash, 0.79%; solubles, 7.4%.

Mixing water for all tests was kept at a temperature of 52°C. The temperature of the batter during mixing was usually about 43°C. The term "total mixing water" used throughout the text and tables refers to the batter composition at mixing, in lb. of water per lb. of dry flour, and includes water present in the flour. Operating data, recovery, and analysis of the products are given in Table I.

The water-to-flour ratio had the greatest effect on gluten protein recovery. When a thick batter was used, an increase of about 10% in protein recovery was attained. In some cases, this increase was accompanied by a decrease in the purity (protein content) of the recovered gluten. When a thin batter is passed through the cutting pump, the gluten is cut into smaller pieces which pass through the screen into the starch fraction. A slight increase in gluten protein recovery is possible by using a finer screen for the primary separation. This increase in recovery was obtained without seriously altering the gluten purity. Holding the thinner batter for an extra hydration period of 30 minutes before processing increased the recovery of gluten protein by almost 10%, with only a slight decrease in gluten purity. A decrease in the speed of the cutting pump when a thick batter was processed improved recovery and purity of the gluten protein.

TABLE I

RESULTS OF PROCESSING FIRST CLEAR FLOUR

EXPERIMENT	A	B	C	D	E	F[a]
Operating data:						
Total mixing water lb/lb dry flour	<——1.17——>			<——1.28——>		
Cutting water lb/lb dry flour	<——1.44——>			<——1.44——>		
Cutting pump speed, r.p.m.	600	600	200	<——600——>		
First screen, mesh	150[b]	80[c]	150	80	150	80
Second screen, mesh	<——100[d]——>			<——100——>		
Products:[e,f]						
Gluten fraction, lb.	14.4	15.4	14.6	12.5	13.1	13.9
Purity, % protein	83.6	77.4	84.8	83.5	82.8	77.5
Starch fraction, lb.	55.2	48.8	51.2	67.6	68.6	56.8
Protein content, %	2.9	3.5	3.1	5.0	4.4	3.6
Wash water solids, lb.	15.8	23.1	18.4	5.7	6.0	13.0
Protein content, %	4.95	3.5	3.3	9.2	8.5	4.5
Recovery:[f]						
Protein recovered in gluten fraction, % of total protein in flour	83.1	82.7	85.0	72.1	75.5	81.9

[a] Holding tank used in this test.
[b] Opening 0.0041 in.
[c] Opening 0.0075 in.
[d] Opening 0.0065 in.
[e] Yield based on 100 lb. of flour (12% moisture).
[f] All figures on moisture-free basis.

The recovery of crude starch was better when the thinner batter was used. However, the protein content of this starch fraction was higher than that obtained in processing the thicker batter, which resulted in decreased gluten protein recovery. The quantity of protein in the wash-water solids was approximately the same in all tests.

Optimum recovery of gluten protein was obtained from this flour when a thick batter was used, the cutting pump was operated at a slow speed, and the finer screen was employed for the primary separation.

Effect of Operating Variables on the Processing of a Second Clear Flour. To confirm the results further and also to examine them in relation to a second clear flour, a study was made testing five processing variables according to a half-factorial experimental design. The five variables considered were: a), water-to-flour ratio, b), temperature of the mixing water, c), speed of the mixer, d), retention time in the mixer, and e), speed of the cutting pump. The experiment was designed chiefly to show which variables are most important in the recovery and purity of the gluten, and for this reason the water-to-flour ratio is higher than would be used for optimum recovery.

In this experiment, a second clear flour having the following analy-

TABLE II

RESULTS FROM A HALF-FACTORIAL EXPERIMENTAL DESIGN USED ON SECOND CLEAR FLOUR IN THE PILOT-PLANT OPERATION OF THE CONTINUOUS BATTER PROCESS

Water-to-Flour Ratio	Temperature of Water to Mixer	Retention Time	Mixer Speed	Cutting Pump Speed	Gluten Protein Recovery, Percent of Total Protein in Flour, MFB[a]	Gluten Purity Percent Protein, MFB
	°C	*minutes*	*rpm*	*rpm*	%	%
1.4	52	18.5	80	200	77.0	71.4
1.4	30	23	40	600	60.0	70.1
1.4	30	18.5	40	200	69.0	72.0
1.55	30	18.5	80	200	69.5	75.3
1.4	30	18.5	80	600	60.4	75.0
1.55	30	23	40	200	68.3	72.8
1.55	52	23	40	600	74.0	68.9
1.4	52	23	80	600	77.4	71.1
1.55	52	18.5	80	600	74.9	73.8
1.4	52	23	40	200	80.3	66.3
1.4	30	23	80	200	72.3	74.6
1.55	52	23	80	200	76.2	70.1
1.55	30	18.5	40	600	57.0	70.2
1.4	52	18.5	40	600	74.3	71.3
1.55	52	18.5	40	200	74.3	67.9
1.55	30	23	80	600	38.4	68.4

[a] Moisture-free basis.

sis (M.F.B.) was used: protein, 17.3%; ash, 1.47%; solubles, 7.45%. Except for the mentioned variables, all operating conditions were held as constant as possible. The cutting water was about 1.42 parts water per lb. of dry flour, and 150-mesh screens were used for the primary separation and for the single washing of the gluten.

The pattern selected for this experimental design and the results of the 16 experiments carried out under it are given in Table II. Results, expressed in terms of gluten protein recovery and gluten purity, show that optimum recovery is achieved under conditions requiring greater energy input. Recovery is increased toward the maximum obtainable by increases in the temperature of the mixing water, decreases in the water-to-flour ratio, and increases in energy input per lb. of batter during the mixing, in that order of importance. Increasing the speed of the cutting pump seriously reduces gluten recovery when the temperature of the mixing water is low or a thin batter is used. When the temperature is optimum for the operating conditions or when a thicker batter is used, an increased pump speed improves the purity of the gluten with little sacrifice in its recovery.

Effects of Ash Content and Physical Characteristics on the Processing of Second Clear Flours. Second clear flours milled from hard wheat possessing different ash contents and varying in some of their physical characteristics were processed in the continuous pilot plant.

TABLE III

RESULTS OF PROCESSING SECOND CLEAR FLOURS IN THE CONTINUOUS PILOT PLANT
(All figures on moisture-free basis)

	Flours				
	A	B	C	D	E
	%	%	%	%	%
Flour composition:					
Protein	16.3	16.1	17.3	19.8	19.8
Ash	0.84	1.26	1.47	1.89	1.95
Solubles	7.04	9.07	7.45	10.93	11.61
Soluble protein	2.77	3.30	3.20	4.06	4.26
Gluten protein recovery, percent of total protein in flour	80.3	79.5	81.5	77.8	77.6
Gluten purity, percent protein	76.7	75.1	74.9	73.8	73.0

TABLE IV

FIXED CAPITAL INVESTMENT FOR A PLANT PROCESSING 50 MILLION POUNDS OF FLOUR ANNUALLY

Land and Improvements	$ 7,500	
Building (100 ft. × 60 ft. × 12 ft.)	72,000	
		$ 79,500
Equipment delivered:		
1 Ribbon blender, about 50 cu. ft., SS., 10 hp.	$10,000	
1 Flour feeder, gravimetric, belt type, capacity 20-600 cu. ft./hr., ½ hp.	8,600	
2 Shakers, gyratory, 5 ft. × 10 ft. screen area, SS, 3 hp.	10,000	
2 Shakers, gyratory, 5 ft. × 7 ft. screen area, SS, 2 hp.	9,200	
1 Shaker, vibrating, 48-in. diam., 1 hp.	3,000	
1 Tank, 3,000 gal., SS	8,000	
1 Tank, agitated, 15,000 gal., SS.	21,000	
1 Bin, flour, 8 ft. × 8 ft. × 6 ft., steel	1,000	
1 Pump, positive displacement, 75 gpm., SS., 5 hp.	3,500	
2 Pumps, positive displacement, 30 gpm., SS., 2 hp.	4,000	
1 Pump, positive displacement, 40 gpm., SS., 3 hp.	2,500	
1 Pump, centrifugal, 60 gpm., SS., 2 hp.	1,160	
2 Pumps, centrifugal, 25 gpm., SS., 1 hp.	2,000	
1 Conveyor, screw, steel, with elevator, 11 hp.	2,000	
1 Conveyor, screw, 6 ft. × 30 in. diam., SS., 3 hp.	5,000	
3 Flowmeters, 0–30 gpm.	1,000	
1 Flowmeter, 0–40 gpm.	350	
1 Flowmeter, 0–12 gpm.	190	
1 Water softener system, 25 gpm., for removing 24,000 grain hardness/hr.	10,000	
1 Water heater, instantaneous type, with control, 25 gpm.	2,000	
		$104,500
Equipment installed, 30% of delivered equipment		31,400
Piping, wiring, instrumentation, 30% of installed equipment		40,700
Contingencies, 15% of installed equipment complete with piping, wiring, etc.		26,500
Engineering fees, 15% of installed equipment complete with wiring, piping, etc.		26,500
Contractor's fees, 4% of installed equipment complete with piping, wiring, etc.		7,000
Fixed capital investment		$316,100

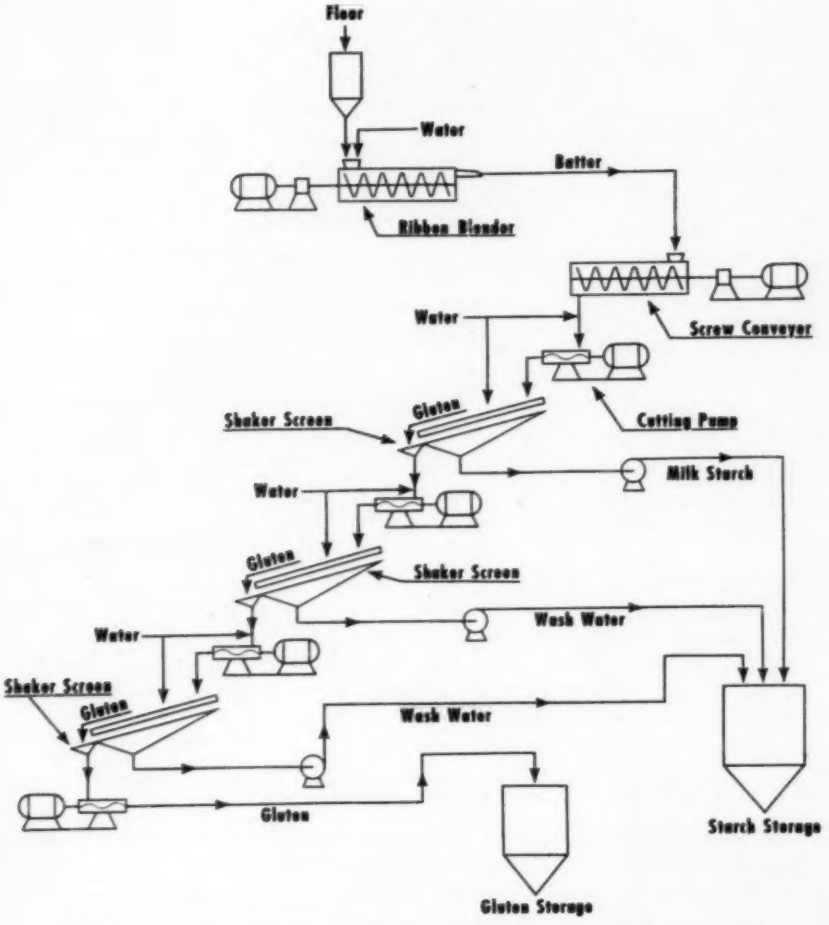

Fig. 1. Flowsheet for processing wheat flour by the continuous batter process.

The chemical composition of five of these flours and test results are listed in Table III.

These flours varied in ash content from 0.84 to 1.95%. The amount of solubles and soluble protein present in the flours increased as the amount of ash increased. The flours were processed under the optimum conditions selected on the basis of previous work. These conditions are: mixing water temperature, 52°C.; total mixing water, 1.15 lb. per lb. dry flour; mixer speed, 66 r.p.m.; retention time in mixer, 18.5

TABLE V

ESTIMATED COST OF PRODUCTION (EXCLUDING RAW MATERIALS) FOR A PLANT WITH A CAPACITY FOR PROCESSING 50 MILLION POUNDS OF FLOUR ANNUALLY

(20 hours per day, 250 days per year)

	ANNUAL COST		CENTS/100 LB. FLOUR PROCESSED
Utilities:			
Electricity 196,000 kw. hr. at $0.015/kw. hr.	$ 2,940		
Water 27,000,000 gal. at $0.075/1,000 gal.	2,025		
Steam 2,500,000 lb. at $0.80/1,000 lb.	2,000		
		$ 6,965	1.4
Labor and supervision:			
3 operators $2.50/hour total, 24 hours/day	15,000		
9 laborers $2.00/hour total, 72 hours/day	36,000		
1 mechanic $2.50/hour, 1/3 time	1,666		
1 superintendent $9,000/year, 1/3 time	3,000		
1 foreman $5,500/year, 1/3 time each shift	5,500		
1 chemist $5,500/year, 1/3 time	1,834		
Overhead	9,450		
		$72,450	14.5
Maintenance:			
Equipment 5%/year	11,830		
Building, 2%/year	1,590		
		$ 13,420	2.7
Fixed charges:			
Depreciation			
Equipment, 10%/year	23,660		
Building, 3%/year	2,160		
Taxes and insurance, 3%/year	9,480		
		$ 35,300	7.0
Miscellaneous operating supplies	$4,500		
		$ 4,500	0.9
		$132,635	26.5

minutes; cutting pump speed, 200 r.p.m.; amount of cutting water, 1.40 lb. per lb. dry flour; the gluten was washed twice.

Purity of the gluten product decreased slightly with an increase in flour ash. Gluten from the flour with the higher ash was more difficult to wash; this was attributed partly to the increased amount of bran present: when ground whole wheat was processed under similar conditions, it was difficult to obtain gluten purity of over 50%. However, in all cases, the crude starch produced was comparable to that from good flours.

Gluten protein recovery also decreased slightly with an increase in ash content; this was caused partially, it is believed, by the increased solubles and soluble protein contents of the flour. Generally, gluten protein recovery of about 80% has been attained when these flours have been processed.

Cost Estimates. Operating and investment costs have been estimated for the plant-scale fractionation of wheat flour to a millstarch slurry (crude starch) and a wet gluten fraction by the continuous batter process. A general flowsheet is given in Fig. 1. Table IV lists the land, building, and equipment needed for a plant processing 50 million lb. of flour annually (second clears, 16% protein, M.F.B.), 20 hours per day, 250 days per year. The total estimated plant investment cost is $316,100, including complete installation of all equipment. This plant would of necessity be operated in conjunction with a starch-refining and a gluten-drying facility, so that some of the equipment and personnel would be shared. In this cost estimate no recovery of the solubles fraction was contemplated even though it amounts to as much as 10% of the total flour used, and any disposition of the solubles is charged to the starch-refining and gluten-drying facility.

The estimated plant production costs for this plant are given in Table V. This cost amounts to $132,635 annually or 26.5 cents per 100 lb. of flour processed. These are plant production costs only and do not include any charges for flour, working capital, profits, income tax, selling expense, or administration.

This processing cost is independent of the type of flour being processed. However, the recovery of gluten protein would vary, depending on the particular flour used. Table VI gives typical gluten and crude starch recoveries from several flours of different types and composition. Since the gluten product is much more valuable than the

TABLE VI

RECOVERY OF GLUTEN AND CRUDE STARCH FROM FLOURS OF DIFFERENT TYPES AND COMPOSITION

TYPE OF FLOUR	PATENT, SOFT WHITE WINTER	PATENT, SOFT RED WINTER	PATENT, HARD RED WINTER	FIRST CLEAR, HARD RED WINTER	SECOND CLEAR, HARD RED WINTER
Composition of flour					
Protein, % MFB[a]	9.2	11.2	14.3	16.6	17.3
Ash, % MFB	0.39	0.34	0.41	0.79	1.47
Gluten recovery					
Recovery from 100 lb. flour (about 12% moisture), lb. MFB	7.0	9.8	13.0	14.6	16.6
Gluten purity					
% Protein, MFB	81.0	81.0	80.7	84.8	74.9
Crude starch recovery (Average 3% protein) recovery from 100 lb. flour (about 12% moisture), lb. MFB	76.5	72.1	73.0	69.6	67.7

[a] Moisture-free basis.

starch product, the most desirable flours to process are those with the highest protein content. Low-grade flours, such as second clears, generally contain greater amounts of protein and are also available at less cost.

Acknowledgment

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PILOT-PLANT PREPARATION OF HYDROXYETHYLATED FLOURS[1]

E. B. Lancaster and V. F. Pfeifer

ABSTRACT

Reaction of ethylene oxide with various cereal flours was accomplished in a simple reactor that prepared 10-lb. batches of hydroxyethyl flours for evaluation studies. One satisfactory method for integrating 2.5% of sodium hydroxide catalyst into the reaction mixture was, first, to prepare a catalyst from 50% sodium hydroxide solution and a small portion of the flour, and then to dry and grind this catalyst before adding it to the bulk of the flour. The reaction rate was increased by using active catalysts which were specially prepared, or by aging the reaction mixture. The reaction of flour with ethylene oxide was exothermic, and the rate also depended upon batch size, temperature, and mol ratio of reactants. Larger batches lost less heat, thus allowing a higher reaction temperature and reaction rate. Reaction rate was decreased by lower ratio of ethylene oxide to flour.

At present, specialty products are available on the market which are produced by subjecting commercial starches to oxidation, esterification, or etherification (1). Recently, the etherification reaction with ethylene oxide has been applied to commercially available grain flours by Rankin, Mehltretter, and Senti (3) to produce materials of improved properties. Because the hydroxyethyl flours were required for use tests, a reactor was designed in which up to 15 lb. per batch could be made. The problems that arose are pointed out, and the means used to solve them are discussed.

Equipment and General Procedure

The reaction vessel consisted of a stainless-steel cylinder with a cone at one end and a flanged cover at the other. The vessel was equipped with a wooden tire near the conical end, and the tire and flange were covered with rubber sheeting, as shown in Fig. 1. The assembly was placed on a large ball-mill drive roll to provide agitation. The reactor rotated at 45 r.p.m. Ethylene oxide gas was introduced intermittently through stainless-steel valves, fittings, and tubing connecting the ethylene oxide cylinder to the cone of the reaction vessel. The pressure gage shown had a range of 15 p.s.i.g. to 30-in. vacuum, and its fitting and Bourdon tube were constructed of stainless steel. The drive motor for the rolls was explosion-proof, and all equipment was grounded.

[1] Manuscript received July 6, 1959. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois, one of the laboratories of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

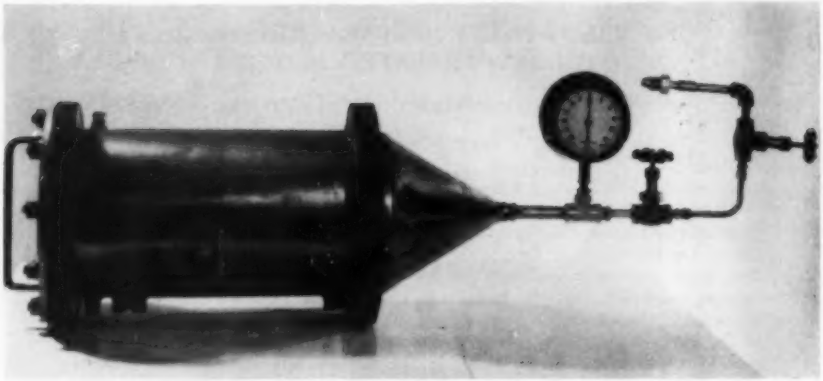

Fig. 1. Reactor used in preparing hydroxyethylated flours.

After the solid reaction mixture was introduced into the vessel through the flanged end, the cover was secured and the vessel was put on the rolls and evacuated. The rolls were started, and the solids were allowed to mix under vacuum for about 15 minutes. The rolls were stopped, nitrogen was introduced to 15 p.s.i.g., and mixing was resumed. The process of mixing under vacuum and then under nitrogen was repeated at least once.

Next, nitrogen was removed to 10 in. of mercury vacuum, leaving about 10 p.s.i.a. in the vessel. Ethylene oxide gas was introduced to bring the pressure to 15 p.s.i.g. or about 30 p.s.i.a.; pressure was periodically increased to that level by adding ethylene oxide at intervals depending on the rate of fall. For most runs, after three additions in the first ten minutes of operation, further increments were added at 20-minute intervals unless excessive heating was observed. To obtain sufficient gas pressure from the ethylene oxide cylinder to give 15 p.s.i.g. in the reaction vessel, it was necessary to heat the cylinder with water at about 140°F. (60°C.). Under these conditions, some ethylene oxide condensed in the connecting tubing, and care was taken during and after disconnection to avoid personal contact as far as possible with the liquid or vapors. The reaction was carried out in a special room equipped with explosion-proof electrical fixtures, including three high-capacity fans to maintain good ventilation.

The reaction was continued until slightly more than the theoretical amount of ethylene oxide had been added. When the pressure indicated that the proper amount had been absorbed, the remainder was removed by evacuating the vessel with a water jet, followed by nitrogen sweeping in the same manner described for preparing the solid

reaction mixture before introducing ethylene oxide.

The solids were weighed before and after the reaction, giving a check of the extent of substitution. From the volume of the vessel, the weight of solid reaction mixture, and the gas law, a factor was obtained to convert total pressure drop during reaction to extent of substitution. Neither method agreed perfectly with the chemical analyses of the final product. In most runs, 7.3 lb. of dry flour yielded 9 to 11 lb. of finished product with a moisture content of about 10%.

Materials and Methods

Screen analyses were carried out by shaking 200 g. of product in a screen set consisting either of 12-, 16-, 30-, and 60-mesh or of 30-, 60-, 100-, and 140-mesh, for 15 minutes. The ethylene oxide substitution was determined essentially by the Lortz modification of Morgan's method (2). Moisture determinations were obtained with the Brabender Moisture Tester[2] although the Karl Fischer or vacuum-oven method gave equal results.

The solid reaction mixture was prepared by adding 2.5% sodium hydroxide equivalent to it. Several methods of doing this were tried, and these are discussed in the next section. The following procedure evolved as the method of choice: to 300 g. of flour in a sigma-blade mixer were added 140 cc. of 50% sodium hydroxide solution over a period of at least 10 minutes. The moist granular mixture was then heated at 140°F. for at least 1 hour in a forced-draft oven. After cooling, the mixture was ground in a Mikro-pulverizer using a 0.020-in. screen. The larger material on the first grind was reground until less than 5% was retained on a 60-mesh screen. An amount of material passing a 60-mesh screen was then used as the catalyst.

The sodium hydroxide content of the catalyst was estimated by adding about 150 ml. of water to a flask containing about 0.25 g. of catalyst accurately weighed. The mixture was titrated with 0.10*N* hydrochloric acid to both a phenolphthalein and then a methyl red end point. The difference was taken as an indication of carbonates, and it was sometimes appreciable.

Results and Discussion

Several lots, grades, and types of wheat flour, two types of corn flour, and a commercially available grain sorghum flour were used in these limited experiments. Any differences among them during hydroxyethylation appear to be minor. Because the flours are currently

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

being evaluated, a complete description of their properties is not given.

In early experiments, several methods of incorporating the sodium hydroxide catalyst into the flour were tried. In one, sodium hydroxide was ground in an attrition mill mounted in a dry-air box, the powder was added to 2 lb. of flour, and the mixture reground. In another experiment, the sodium hydroxide was ground in a mortar and pestle, screened through 140-mesh screen, and added to the flour. Despite all precautions, appreciable quantities of large, pink, jellylike particles were found in the final product. The minimum amount was about 0.5% over a 16-mesh screen. These particles were analyzed and found to contain about double the sodium hydroxide and moisture concentrations of the remaining product, and about one-fourth of the protein content.

Because it was most difficult to mix dry sodium hydroxide and flour into a relatively homogeneous system without particle formation, methods of preparing a better catalyst and of reducing particle size were investigated. The biggest problem encountered was overheating, followed by browning and plasticizing in the materials. Soda ash can be used to prepare a high-sodium mixture very successfully, but the rate of reaction of the mixture of dry soda ash, flour, and ethylene oxide in one run was very slow. Finally, it was found that considerable 50% sodium hydroxide could be added to flour without appreciably developing plasticity in the flour, but that at a certain stage of the addition, granulation occurred with a consequent swelling of the mass.

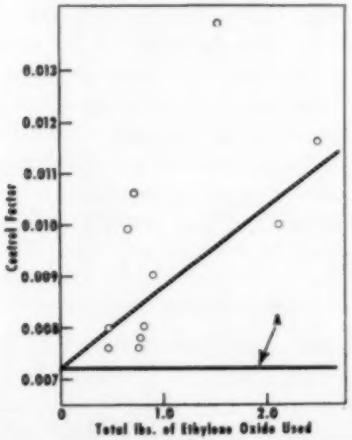

Fig. 2. Relation of control factor, the pounds of ethylene oxide actually used for each p.s.i.g. drop in pressure in the reactor, to the total amount used for the batch. *A* is the relation computed from the reactor volume.

Some ammonia is liberated near the end of the addition, and heat is liberated. The finished mixture is a mass of relatively soft aggregates, 80% of which pass a 30-mesh screen. This material, when fresh, could be readily ground in a "pug" mill, but attrition or hammer mills cause it to plasticize.

The next series of experiments was run using catalyst ground in a pug mill to pass a 30-mesh screen. The results are shown in Figs. 2 and 3. The horizontal line in Fig. 2 is the computed factor for converting total drop in pressure to weight of ethylene oxide introduced into the reactor; whereas the diagonal line represents actual results using a standard operating procedure in which a 9-p.s.i.g. increase in reactor pressure was accomplished in a fixed time (about 10 seconds). Deviation of the control line from its predicted horizontal position is a matter of relative rates of addition and reaction. As will be shown, the reaction rate increases for extended runs, so that the net effect is to increase the control factor as higher amounts of total ethylene oxide are used. Once this curve is established, substitutions can be carried out to within 0.5% of the desired values.

Figure 3 indicates the swelling of the catalyst particles. Since all the catalyst passed a 30-mesh screen, the presence of particles over 30-mesh can probably be interpreted as particle-size growth in the catalyst.

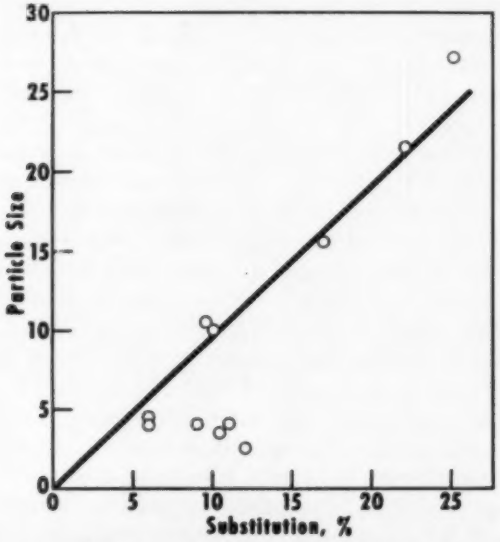

Fig. 3. Particle size, as weight percent of particles in the hydroxyethylated flours retained on a 30-mesh screen, increased with percentage ethylene oxide substitution.

These large particles appear as translucent, insoluble spheres in pastes made in the usual way, and in some applications these particles would not be permissible. The cluster of low points probably represents a finer catalyst owing to the slower addition of 50% sodium hydroxide to the flour. One of these points is for a run made with preground solid sodium hydroxide.

During the experiments, it was noticed that the catalyst particles became harder on air-drying and that the catalyst lost moisture with little gain in carbonate. The dried catalyst was readily ground in a hammer mill to pass a 60-mesh screen. When this catalyst is used immediately, the reaction rate is reduced to about half, but when it is mixed with the flour and the mixture is allowed to age for over 2 days, the reaction proceeds as usual. In any case, there are no particles in the product larger than 30-mesh, and pastes made from hydroxyethylated flours seem to be greatly improved.

To avoid long aging times the catalysts were prepared by oven-drying the fresh material, although some yellowing occurred and the dried material was somewhat softer than the air-dried catalyst. If a vacuum cleaner bag was used as a receiver to prevent moisture condensation on the ground product, the oven-dry catalyst could be ground in a hammer mill. It performed well in two runs. In a third run when the catalyst was dried for an intermediate time, subjected to the maximum grinding action (i. e., two repasses of about 30% of the first grind), and stored in a covered glass jar for 1 day before use, it produced a lower rate of reaction. Typical examples using various methods of making the catalyst for 12%-ethylene oxide-substituted flours are presented in Table I.

Besides the observed effect of catalyst amount and condition on the rate of reaction, limited work has shown that pressure and batch-size control the rate also, apparently through a complex relationship with temperature. The pressure, of course, determines the concentration of ethylene oxide in the nitrogen-containing atmosphere and, therefore, the ratio of reactant to flour at constant batch-size. Reduction in batch-size at constant pressure increases the amount of ethylene oxide with respect to the flour but also increases the efficiency of heat exchange through the walls of the reaction vessel. The reaction is definitely exothermic, and the product becomes quite warm with extended reactions and active catalysts with an 8-lb. batch-size. The smaller batches react somewhat slower than larger ones. A typical course of an active reaction is presented in Fig. 4 when the usual procedure is followed. The slopes of the diagonal lines represent the rates of absorption of ethylene oxide by the flour; the height of the lines above the horizontal

TABLE I

COMPARISON OF SODIUM HYDROXIDE CATALYST PREPARATION METHODS

METHOD	PARTICLE SIZE[a]	FINAL RATE	PRODUCT PARTICLE SIZE
		psig/minute at 15 psig	*% over 60-mesh*
Ground dry by machine and mixed	....	..	26
Ground dry by hand, screened, and mixed	....	..	14.5
Mixed wet, pug ground	Coarse	0.2–0.5	15–30
Mixed wet, air dried, hammer-ground, aged	Fine	0.6	7.5
As above, not aged	Fine	0.2	7
As above, not aged hammer-ground, not aged	Fine	0.6	7

[a] Coarse — 100% through 30-mesh. Fine — 100% through 60-mesh, about 65% through 140-mesh.

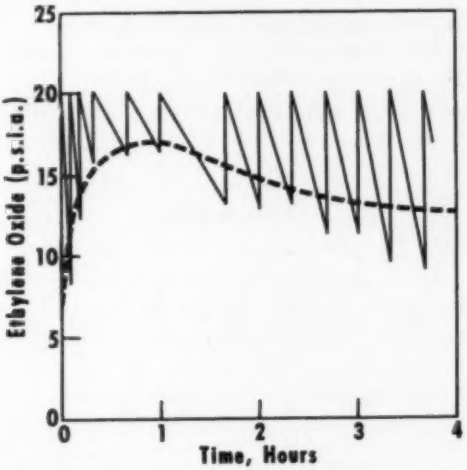

Fig. 4. Reaction rate at incremental adjustment to constant pressure. The pounds absolute pressure represent the weight of ethylene oxide present at any time in the reactor, which was refilled to 20 p.s.i.a. at about 20-minute intervals. The dotted line suggests the reaction with constant feed.

axis is an indication of driving force. The broken curve suggests the course that might be expected with a constant rate of ethylene oxide addition.

Although no attempt was made to analyze the degree of heterogeneity in the reaction product, there was one other indication, besides the swelling around the catalyst particles, that nonuniform reaction oc-

curs. In the "dead" spaces of the reactor (at the center of the two ends) occasionally the flour was found to be caked and, near the ethylene oxide inlet, to be somewhat pasty in character. The cake, if dry, was quite friable and could easily be blended back into the product.

Factors in Plant Design. The important consideration in the large-scale production of hydroxyethyl flours will be the control of the operation under safe conditions. The toxic and explosive properties of ethylene oxide are well known, and information on the precautions necessary in handling it is usually available from the distributor.

A practical reactor must be designed to bring together a solid and a gas and, probably more important, to remove heat from the solid without introducing surfaces to the gas which would be cold enough to cause condensation. Some provision may be necessary for heating the mixture at the start of the reaction.

Control of reaction by pressure drop is uncertain; an integration method such as continuous weight measurement of the ethylene oxide input would be preferred. Overheating, which would cause starch gelation, must be avoided, so that a good control is needed to adjust the feed rate of the gas by temperature change as well as by pressure limitation. Pressure control is necessary to avoid exceeding the explosive limits or condensation point of ethylene oxide gas in the vapor phase of the reaction.

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GRAIN STORAGE STUDIES

XXIX. Effect of Invasion by Individual Species and Mixtures of Species of Aspergillus upon Germination and Development of Discolored Germs in Wheat[1]

G. C. Papavizas[2] and C. M. Christensen[2]

ABSTRACT

Wheat samples were surface-disinfected and inoculated with spores of *Aspergillus amstelodami, A. ruber, A. restrictus,* and *A. candidus,* alone and in combinations, stored at 25°C. and at relative humidities of 75, 80, and 85% (14.7–14.9, 16.0–16.4, and 17.0–17.2% moisture content, respectively) and after 1 to 5 months tested for germination, discolored germs, and numbers and kinds of fungi. Inoculation with mixtures of species did not result in greater loss of germination or development of more germ damage than did inoculation with the most pathogenic species alone. Samples of durum, hard red spring, hard red winter, and white wheats were inoculated with spores of *A. amstelodami* and *A. candidus,* stored 3 months at 25°C. and 80% relative humidity, and similarly tested. Damage, as evaluated by reduction in germination and increase in discolored germs, was greater in the hard red winter and white wheats than in the durum and hard red spring wheats. The noninoculated controls in all tests remained free or almost free of fungi, retained a high germination percentage, and developed little or no germ damage.

It is not unusual, in culturing wheat that in storage has developed dark brown or black germs (in the grain trade designated "sick" or "damaged"), for several species of *Aspergillus* to grow from a single germ. The work here reported was undertaken to determine whether a combination of two or more species of storage fungi isolated from such damaged kernels might be more injurious than each species alone. Two of the individual species of *Aspergillus* also were inoculated onto samples of four different classes of wheat to determine whether there might be differences among them in susceptibility to invasion and to damage by these fungi.

Materials and Methods

Wheats. In the tests comparing mixtures vs. individual species of *Aspergillus,* a hard red spring wheat grown in the research field at St. Paul was used. In the tests comparing the different classes of wheats, the same hard red spring wheat was used plus durum wheat also grown

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on the research field at St. Paul, a white wheat grown on irrigated land in eastern Oregon, and a hard red winter wheat from a commercial storage bin in Omaha, Nebraska. The hard red spring, durum, and white wheats were very low in storage molds; the winter wheat had a moderate infection by storage molds.

Moisture content was determined by the 2-stage, air-oven method specified by *Cereal Laboratory Methods* (1) and is expressed on a wet-weight basis.

Preparation and Storage of Samples. Portions comprising 200 g. each were submerged and shaken for 2 minutes in 1% sodium hypochlorite, rinsed in sterile water, and dried to 11.5% moisture content. Previous work (7) indicated that this treatment did not measurably affect germinability or susceptibility of the grain to subsequent invasion by storage fungi. The moisture content was adjusted to the desired levels by adding sterile water to the controls and a suspension of spores in water to those inoculated, after which the grain was kept 5 days at 4°C. and shaken occasionally to permit uniform distribution of the water.

Portions of 10 g. each were placed in sterile 25-ml. plastic vials; the open ends of the vials were covered with a double layer of sterile cheesecloth; and the vials were placed, covered end down, on the perforated plate of a desiccator containing a saturated salt solution which maintained a relative humidity in equilibrium with each moisture content used. Saturated solutions of sodium chloride, ammonium sulfate, and potassium chloride were used for relative humidities of 75, 80, and 85%, respectively (8). Samples were stored at 25°C. and tested after 1 to 5 months.

Inoculum consisted of spores of the various species of *Aspergillus* suspended in sterile distilled water to which one part in 10,000 of Tween 20[3] had been added as a wetting agent before the water was autoclaved. The spore suspensions were so adjusted that the amount added to each sample of grain contained 20,400 ± 500 spores, as determined by counting 10 samples of each in a Spencer hemacytometer. The mixtures contained the same total number of spores, evenly divided among the species making up each mixture. The fungi used were *A. amstelodami* (Mang.) Thom & Church; *A. ruber* (Spieck. & Bremer) Thom & Church; *A. restrictus* G. Smith; and *A. candidus* Link.

Germination was measured by incubating 100 or 200 kernels on moist paper at 18°C. for 6 days.

Germ color was evaluated by removing the pericarps over the

[3] Polyoxyethylene sorbitan monolaurate, a product of Atlas Powder Co., Wilmington, Delaware.

embryo of 100 or 200 kernels and examining the embryo with a stereoscopic microscope, using a magnification of × 10. Only dark brown or black embryos were rated discolored or damaged.

Fungus Invasion. One hundred kernels were shaken for 2 minutes in 1% sodium hypochlorite, rinsed in sterile water, and cultured on malt agar containing either 7.5% sodium chloride for detection of most of the species of *Aspergillus*, or 20% sodium chloride, for the detection of *A. restrictus*. There is considerable evidence that these media serve fairly well for this purpose (2,3,4,5,6).

Mold Count. Five grams of grain were comminuted 1 minute in 500 ml. of 0.2% sterile agar solution in water; successive dilutions were made in the same medium; and replicate 1-ml. portions of one or more dilutions were cultured in malt agar containing 7.5% sodium chloride. Colonies were counted and identified after 5–10 days.

Results

The results of tests on wheat inoculated with spores of individual species and mixtures of species of *Aspergillus* and stored 1 month at 85% r.h., 2 months at 80% r.h., and 5 months at 75% r.h. are given in Tables I and II. Mixtures of species did not reduce germination or increase germ damage more than the most pathogenic member of the mixture. At relative humidities of 80 and 85% *Aspergillus candidus* alone or in mixtures resulted in greater loss of germination, and in the development of more discolored germs, than did *A. restrictus* alone or in mixtures. This would be expected, since a moisture content of 16–17% (in equilibrium with a relative humidity of 80–85%) is known

TABLE I

GERMINATION, GERM DISCOLORATION, AND SURFACE-DISINFECTED SEEDS YIELDING FUNGI OF WHEAT NOT INOCULATED AND INOCULATED WITH SPORES OF INDIVIDUAL SPECIES AND MIXTURES OF SPECIES OF ASPERGILLUS[a]

(Grain stored 1 month at 25°C. and 85% relative humidity; 17.0–17.2% moisture content)

INOCULUM	GERMINATION	DARK GERMS	SURFACE-DISINFECTED KERNELS YIELDING ASPERGILLUS
	%	%	%
Control — not inoculated	93	0	1
A. candidus	20	75	100
A. ruber	32	47	95
A. restrictus	34	57	100
A. candidus + *A. ruber*	25	68	99
A. candidus + *A. amstelodami*	26	72	100
A. candidus + *A. restrictus*	19	67	100
A. candidus + *A. ruber* + *A. restrictus*	25	59	100

[a] Each figure is an average of four replicates of 100 kernels each.

TABLE II

GERMINATION, GERM DISCOLORATION, SURFACE-DISINFECTED SEEDS YIELDING FUNGI, AND MOLD COUNT OF WHEAT NOT INOCULATED AND INOCULATED WITH SPORES OF INDIVIDUAL SPECIES AND MIXTURES OF SPECIES OF ASPERGILLUS[a]

INOCULUM	GERMINATION	DARK GERMS	SURFACE-DISINFECTED KERNELS YIELDING ASPERGILLUS	COLONIES PER GRAM OF GRAIN (IN THOUSANDS)			
				A. candidus	*A. amstelodami*	*A. ruber*	*A. restrictus*
	%	%	%				
A. Grain stored 2 months at 25°C. and 80% relative humidity (16.0–16.4% moisture content)							
Control — not inoculated	90	0	0		— not determined —		
A. candidus	25	70	100	5750	0	0	0
A. amstelodami	38	40	81	0	1600	0	0
A. ruber	34	57	91	0	0	750	0
A. restrictus	40	53	100	0	0	0	4000
A. candidus + *A. amstelodami*	42	49	98	2350	1200	0	0
A. candidus + *A. ruber*	34	60	94	3200	0	1100	0
A. candidus + *A. restrictus*	31	58	100	400	0	0	4000
A. candidus + *A. amstelodami* + *A. restrictus*	27	63	100	1000	400	0	4300
B. Grain stored 5 months at 25°C. and 75% relative humidity (14.7–14.9% moisture content)							
Control — not inoculated	89	4	6		— not determined —		
A. candidus	68	11	95	0	0	0	0
A. amstelodami	47	38	100	0	1250	0	0
A. ruber	61	22	100	0	0	250	0
A. restrictus	44	40	100	0	0	0	2200
A. candidus + *A. amstelodami*	54	34	100	200	1000	0	0
A. candidus + *A. ruber*	56	23	99	300	0	1200	0
A. candidus + *A. restrictus*	51	41	100	150	0	0	2600
A. candidus + *A. amstelodami* + *A. restrictus*	45	35	97	200	450	0	3350

[a] Each figure is an average of four replicates.

to be favorable to the growth of *A. candidus,* whereas a moisture content of 14.7–15% (in equilibrium with a relative humidity of 75%) is about the minimum for *A. candidus* but very favorable for *A. restrictus* (5). This is shown in Fig. 1.

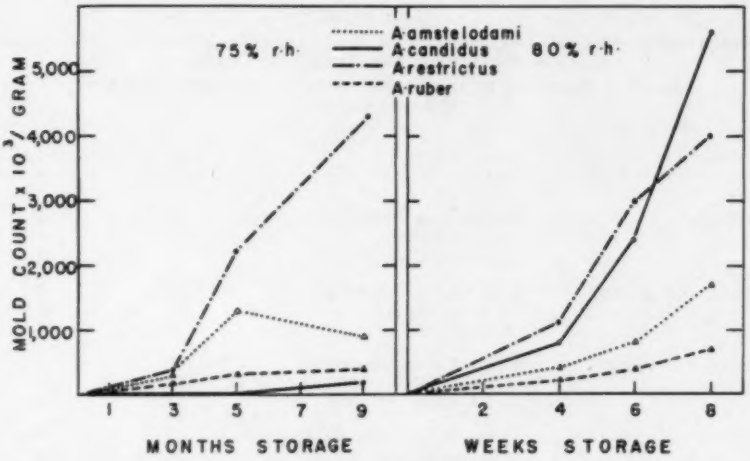

Fig. 1. Numbers of colonies of four species of fungi from wheat inoculated with them and stored at 75% and 80% r. h.

The noninoculated grain stored for 1 and 2 months at the higher moisture contents remained free of fungi, retained a germination of 90% or more, and developed no germ damage. The control at 75% relative humidity, stored 5 months, yielded storage fungi from 6% of the surface-disinfected kernels (apparently from inoculum that was not eliminated by the surface-disinfection treatment at the beginning of the tests), and the germs of 4% were dark, although the germination was still 89%. There is little doubt that the decrease in germination and increase in germ damage of the inoculated grain in these tests was caused directly by storage fungi, since in the absence of the fungi the grain remained sound and of high germination. This agrees with the results of numerous other tests (3,4,5,6). The greatest difference between noninoculated and inoculated samples was at 85% relative humidity (Table I) where the controls retained a germination of 93% and had no discolored germs, whereas those inoculated with *A. candidus* germinated only 20%, and 75% of the kernels had dark germs.

Effect of Aspergillus candidus *and* A. amstelodami *on Germination and Development of Discolored Germs in Four Classes of Wheat Stored 3Months at 80% r.h. and 25°C.* The major results are given in Table

III. Using percent germination and percent discolored germs as criteria of damage, the wheats would be rated from least to most damaged in the order: durum, hard red spring, hard red winter, and white wheat – with the single exception that *A. amstelodami* caused more damage to

TABLE III

GERMINATION AND GERM DISCOLORATION OF FOUR CLASSES OF WHEAT, NOT INOCULATED AND INOCULATED WITH TWO SPECIES OF ASPERGILLUS[a]

(Stored 3 Months at 25°C. and 80% Relative Humidity; 16.0–16.4% Moisture Content)

WHEAT	INOCULUM	GERMINATION	DARK GERMS
		%	%
Durum	Control – not inoculated	87	6
	A. amstelodami	65	16
	A. candidus	45	21
Hard red spring	Control – not inoculated	88	2
	A. amstelodami	42	25
	A. candidus	32	57
Hard red winter	Control – not inoculated	80	6
	A. amstelodami	23	69
	A. candidus	17	73
White	Control – not inoculated	95	0
	A. amstelodami	45	31
	A. candidus	6	79

[a] Each figure is an average of four replicates of 100 kernels each.

hard red winter than to white wheat. The noninoculated samples remained high in germination and low in germ damage and, where a few discolored germs were found in the controls, the kernels were also infected with storage fungi, apparently from inoculum that had not been eliminated by the surface disinfection at the beginning of the tests.

Discussion

Evidently none of the mixtures of storage fungi used in these tests were appreciably more damaging than was the most pathogenic member of the mixture when used alone. The results are subject to the limitation that only a single isolate of each species was tested. There may be great differences among isolates, as has already been shown for *A. candidus* (5), and it is possible that mixtures of isolates other than those tested, or of species other than those tested, would reveal that certain combinations are more damaging than single isolates alone. However, if that were a general phenomenon, one would expect to have found at least some indication of it in tests such as those reported here. It seems fair to conclude that mixtures of the fungi common in grain stored for some months at moisture contents of 15–17% are not

likely to be more damaging than individual species. In wheat stored at a moisture content of 14.7–15.0% and a temperature of 25°C. for several months, members of the *A. glaucus* group, such as *A. amstelodami, A. ruber,* and *A. restrictus,* are capable of causing great reduction in germination and severe germ damage.

Hard red winter and white wheats appeared to be more susceptible to damage under the conditions of the tests than either durum or hard red spring wheat. The durum and hard red spring wheats were both of very high quality, typical of that encountered in certified seed, whereas the winter wheat was from a commercial parcel that had been in storage for some time and had been exposed to some invasion by storage molds. This, and not the wheat class, as such, may have been mainly responsible for the differences found. The white wheat, however, was of essentially the same high quality as the hard red spring and durum wheats, yet sustained appreciably more damage. It is at least possible, but by no means proven, that white wheat stored under conditions favorable for the development of storage fungi may be inherently more subject to damage than durum or hard red spring wheats stored under the same conditions. Evidence from tests now in progress at least suggests that different samples of the same variety of hard red spring wheat may differ greatly in susceptibility to invasion by storage fungi, as a result of factors not yet known.

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FORMATION OF STARCH IN WHEAT GRAIN[1]

ROY L. WHISTLER AND J. R. YOUNG

ABSTRACT

Wheat plants were either injected with randomly labeled sucrose-C^{14} or allowed to take up radioactive carbon dioxide in the light. In both groups of plants, the amylose became labeled much sooner than the amylopectin, clearly indicating that the path to amylose is more direct than the path to amylopectin. Although no evidence is available to suggest that amylose is not converted to amylopectin while these polysaccharides are being formed, the data suggest that amylose is not extensively converted to amylopectin once it has become incorporated into the granule.

In plants injected during daylight, radioactivity could be detected in the starch within 4 hours. Those injected during hours of darkness produced little or no labeled starch in 4 hours. Since no activity was found after 4 hours in the heads of plants injected at 11 p.m., translocation of carbohydrates to the head must have stopped during this period of the night.

Wheat plants which were grown under different day lengths had starch with the normal 25% amylose content. This indicates that the relative rate of formation of amylose to amylopectin remains the same whether the plant is in light or darkness.

Enzymatic conversion of amylose to amylopectin has been well demonstrated *in vitro* and excellent reviews are available (5,15). Recently two workers have doubted that amylose is converted to amylopectin during starch synthesis in the plant, and each has proposed a different mechanism for the simultaneous formation of amylose and amylopectin from a common precursor (7,15). Data obtained by examinations of the radioactivity of amylose and amylopectin from wheat starch 1 or 2 months after injection of the plant stems with glucose-1-C^{14} have been interpreted as evidence that amylopectin is formed from amylose (11).

The present paper reports the relative extent to which amylose and amylopectin incorporate carbon-14 when sucrose-C^{14} is injected into the wheat stem and when wheat plants are exposed to a $C^{14}O_2$ atmosphere in the light.

Amylose content of wheat starch produced under different daylengths is reported.

Material and Methods

Wheat Plants Used. 1. Field: The wheat plants (Vermillion variety) injected with sucrose-C^{14} were grown in a filler plot on the Purdue Agronomy Farm.

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2. Greenhouse: Another variety of wheat, Monon, was planted November 18, 1959, in flats and was vernalized in a cold frame from November 24, 1958, until January 26, 1959. The plants were transplanted to 4-in. clay pots and grown in the greenhouse using supplemental light (10 p.m. to 2 a.m.). On March 26, 1959, eight plants were blooming and were moved to a room having lights controlled by a time clock. Half were grown to maturity with alternating periods of 2 hours light and 1 hour dark. The other four plants were subjected to alternating periods of 16 hours light and 8 hours dark. All were harvested at maturity on April 27, 1959; the starch was isolated, and the amylose content was determined. Thirty plants that bloomed between April 1 and April 7 were arranged into five samples in such a way that each sample had the same average blooming date and were given $C^{14}O_2$ on April 29, 1959.

3. Controlled climate: Wheat plants (Vermillion variety) were grown in the controlled-climate laboratory at 70°F. with 12-, 16-, and 20-hour day lengths. The starch was isolated and the amylose content was determined.

Administration of Carbon-14 to Wheat Plants. 1. Field: Randomly labeled sucrose-C^{14} (approximately 120 μc. per mg.) was prepared from a canna leaf surrounded by an atmosphere containing $C^{14}O_2$ according to the method of Putman, Hassid, Krotkov, and Barker (14). A 100-μl. aliquot of an aqueous solution of the sucrose-C^{14} was injected into the hollow part of each plant stem according to the method of Bilinski and McConnell (4). Plants were in the early dough stage about 16 days after flowering when injected with sucrose-C^{14}.

On June 26, 1958, approximately 17.5 μc. were given each plant. Those injected at 3 a.m. were harvested at 7 a.m.; those injected at 7 a.m. were harvested at 11 a.m.; and so on, at 4-hour intervals for a 24-hour period (all times are Central Standard Time). When harvested the heads were placed in plastic freezer bags and immediately frozen with dry ice.

On June 12, 1959, one series of injections started at 9 a.m. was completed by 10:45 a.m. The first eight samples received about 18.2 μc. per plant (ten plants per sample). The last five samples in this series received only 12.2 μc. per plant. Samples were harvested 0.25, 1, 2, 4, 9.6, 11.4, 13.3, 17.6, 56.2, 104, 147, and 336 hours after injection. The last sample was binder-ripe. Another series of four samples was injected from 6:30 p.m. to 7:05 p.m. on June 12. Each plant received only 7.9 μc. of sucrose-C^{14} of undetermined specific activity. The specific activity was probably not more than half that of the sucrose-C^{14} used in the other experiments. These samples were harvested 1, 3, 5,

and 9 hours after injection. The first sample was harvested at sunset.

2. Greenhouse: Thirty potted Monon wheat plants were placed in a 3 by 3 by 3-ft. frame covered with polyethylene plastic film 25 days after flowering (late dough stage). Two mc. of $BaC^{14}O_3$ were placed with 10 ml. water in a 125-ml. Erlenmeyer flask which was secured to the top of a magnetic stirrer. A thin-walled 1-ml. sealed tube of 85% lactic acid was placed in the flask with the $BaC^{14}O_3$. After the plastic cover was in place (11 a.m.) the magnetic stirrer was started from the outside, releasing the lactic acid into the $BaC^{14}O_3$. After 1 hour's stirring, the plastic was removed and the first sample (six plants) was taken. Another sample was taken at 3 p.m. and the other three samples were moved to a darkened hood. These last three samples were harvested at 4, 5, and 7 p.m.

Isolation and Fractionation of Starch. The grain was threshed by hand, while the heads were still frozen. The grains were crushed, a few at a time, in a glass mortar with a glass pestle. Small quantities of ice-cold 1% aqueous solution of sodium chloride were added as the grains were ground. The starch was washed through No. 25 bolting cloth. Vigorous shaking of the starch suspension with equal quantities of the sodium chloride solution and pentanol caused the protein to coagulate and separate at the interface. By repeated washing and sedimentation, the starch was separated from other impurities. The starch from the 1958 crop was fractionated immediately. The starch from the 1959 crop was stored in the freezer under 1% sodium chloride solution until fractionated.

The starch was fractionated without drying by a modification of the anaerobic leaching procedure of Baum, Gilbert, and Wood (2). In this fractionation, finely dispersed bubbles of nitrogen were passed for 30 minutes through 100 ml. of 1% aqueous sodium chloride solution containing about 0.5 g. of starch. Traces of oxygen were removed from the nitrogen by passing it through three traps of Fieser's solution (8) followed by a trap of concentrated sulfuric acid. Then the mixture was heated in a boiling water bath for 10 minutes followed by cooling to room temperature. The amylopectin formed a clear gel after 20 minutes' centrifugation at $2,400 \times G$. The amylopectin was washed five times with aqueous sodium chloride solution and redispersed as before. After five more washes with water, the amylopectin was suspended in about 15 ml. of water and poured into a blender containing 200 ml. of methanol. The precipitated amylopectin was washed with methanol and dried. Amylopectin thus prepared still contained 4–5% amylose as determined by electrometric iodine titration (1). The amylose solution was filtered through a medium sintered-glass

filter. Butanol in the critical amount (5% by weight) according to the method of Hiemstra, Bus, and Muetgeert (9) was added to the solution at room temperature. After overnight standing, the amylose butanol complex was removed as a tightly packed white gel by centrifugation at 2,400 × G. The supernatant solution was decanted and the amylose-butanol complex was dissolved in distilled water at room temperature. The second and third crystallizations with butanol occurred in 1 hour or less after the butanol was added. After three such crystallizations, the complex was dissolved in 30 ml. of water and slowly poured into a blender containing 250 ml. of methanol. The precipitated amylose was washed three times with methanol and dried. Amylose so prepared bound the same amount of iodine as a standard corn amylose prepared by the usual procedures. The starch of the 1959 crop was fractionated by the same procedure, except that a phosphate buffer (10) was substituted for the sodium chloride solution in the dispersions.

Determination of Radioactivity. Starch, amylose, and amylopectin were oxidized to carbon dioxide and plated as barium carbonate by a modification of the method of Claycomb, Hutchens, and Van Bruggen (6). An excess of sodium hydroxide was placed in the receiver and the titrations were omitted, since aliquots were not taken and dilutions were not made. The sample weight was chosen to obtain about 30 mg. per cm.^{2} on an aluminum planchet 1.25 in. in diameter. Activity was measured in a thin-window gas flow Geiger tube (D 47 Nuclear, Chicago). Appropriate corrections for background and coincidence were applied.

Determination of Amylose Content of Starch. Grain from the wheat grown under different day-lengths was dry and was soaked 24 hours in water before grinding. The rest of the isolation was as previously described. Starch was washed with 85% methanol and extracted 24 hours with 85% methanol in a micro-Soxhlet apparatus. The starch was then dried and moisture was determined. Amylose was determined by a modification of the colorimetric method of McCready and Hassid (12). A starch sample containing 100 mg. of dry starch was weighed into a 250-ml. volumetric flask, 100 ml. of distilled water were added and the material was autoclaved 2 hours at 15 lb. per sq. in. Upon cooling, 25 ml. of 1*N* potassium hydroxide solution were added and the flask swept with nitrogen and stored, tightly stoppered, in the refrigerator. After room temperature was reached, water was added to bring the amount to 250 ml. Five milliliters were pipetted into a 200-ml. volumetric flask and 100 ml. of water added. Hydrochloric acid, 0.1*N*, was added to adjust pH to approximately 5 (about 5 ml.). Two

milliliters of iodine solution were added (0.2% I_2, 2.0% KI) and the solution was made to volume with water. The blank was made the same but without starch. The solution was read at 610 $m\mu$ in a spectrophotometer. Amylose content was determined by reference to a calibration chart previously prepared.

Results

Specific activities of wheat starch amylose and amylopectin from plants injected at 9 a.m. June 12, 1959, and harvested after various periods are shown in Table I. The activity of the starch and fractions rose very rapidly during the first 9 hours after injection. Smaller

TABLE I

SPECIFIC ACTIVITIES OF STARCH, AMYLOSE, AND AMYLOPECTIN FROM WHEAT INJECTED WITH SUCROSE-C^{14} 9 A.M. JUNE 12, 1959

(c/m $BaCO_3$ infinite thickness)

TIME AFTER INJECTION	STARCH	AMYLOSE	AMYLOPECTIN	RATIO OF SPECIFIC ACTIVITY, AMYLOSE/AMYLOPECTIN
hours				
0.25	...	0	0	...
1.0	10	26	2	...
2.0	154	403	15	27.0
4.0	1,580	2,780	394	7.0
9.6[a]	7,770	12,700	2,370	5.4
11.4	9,150	16,000	3,830	4.2
13.3	9,500	13,900	5,330	2.6
17.6[b]	7,530	10,700	2,860	3.7
56.2	15,700	16,900	7,700	2.2
104.0	15,300	18,200	8,100	2.2
147.0	14,200	16,600	9,700	1.7
336.0	15,400	17,300	11,900	1.5

[a] Sample taken at sunset.
[b] Sample taken 30 minutes before sunrise.

changes in activity would not be apparent because equivalent amounts of radioactivity were not incorporated into each sample. The ratios of specific activity of amylose to that of amylopectin are also given in Table I. These values are free from variations due to different amounts of carbon-14 incorporated by each sample. The ratio of specific activity of amylose to amylopectin dropped very rapidly during the first 24 hours and then decreased only slightly during the next 2 weeks.

The specific activities of starch, amylose, and amylopectin from plants injected at 6:30 p.m. June 12, 1959, and harvested after definite periods are shown in Fig. 1. Here, too, the amylose is labeled much more quickly than the amylopectin.

Similar data are given in Fig. 2 for starch from plants exposed to

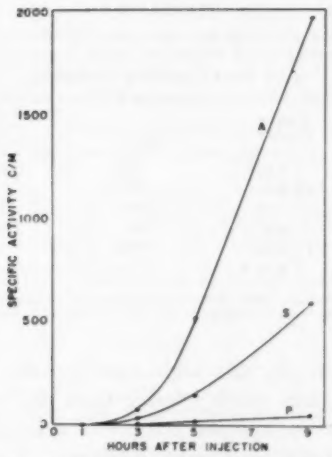

Fig. 1. Specific activity of (S) starch, (A) amylose, and (P) amylopectin from wheat injected with sucrose-C^{14} at 6:30 p.m. June 12, 1959.

an atmosphere containing $C^{14}O_2$. As previously observed, the amylose became labeled before the amylopectin.

Specific activities of wheat starch and its main fractions isolated 4 hours after plants were injected with sucrose-C^{14} at different times of the day are shown in Table II. Plants injected at 11 p.m. and harvested at 3 a.m. the following day had no measurable amount of radioactivity in the starch. In fact, no detectable amount of radioactivity

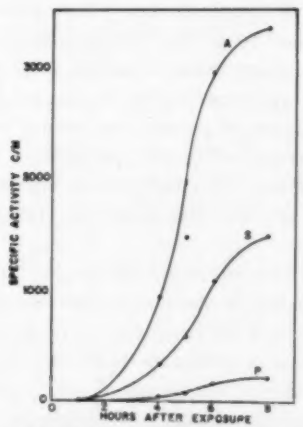

Fig. 2. Specific activity of (S) starch, (A) amylose, and (P) amylopectin from wheat exposed to $C^{14}O_2$ at 11 a.m. April 29, 1959.

TABLE II

SPECIFIC ACTIVITY OF STARCH, AMYLOSE, AND AMYLOPECTIN FROM WHEAT HARVESTED 4 HOURS AFTER INJECTION WITH SUCROSE-C^{14}
(c/m $BaCO_3$ infinite thickness)

TIME OF INJECTION	TIME OF HARVEST	SPECIFIC ACTIVITY		
		Starch	Amylose	Amylopectin
3 a.m.	7 a.m.	39	105	2
7 a.m.	11 a.m.	259	677	16
11 a.m.	3 p.m.	389	606	61
3 p.m.	7 p.m.	482	1040	110
7 p.m.	11 p.m.	10	43	0
11 p.m.	3 a.m.[a]	0	1	0

[a] June 27, 1958. All others June 26, 1958. Beginning of morning twilight 3:45 a.m., sunrise 4:18 a.m., sunset 7:23 p.m., end of evening twilight 7:56 p.m. Maximum temperature June 26, 72°F. Minimum temperature June 27, 54°F.

entered the heads during this night-time period. Specific activity of amylose was in all cases much greater than that of the amylopectin.

It is evident that the specific activity of the starch is greater than the activity of the fractions would indicate.

Amylose content of starch from Vermillion variety wheat grown in the controlled-climate laboratory under 12-, 16-, and 20-hour days was 25.5, 23.8, and 23.5%, respectively. Amylose content of Monon variety wheat starch, produced with lights on 2 hours, off 1 hour, and on 16 hours, off 8 hours, was 25.5 and 25.0%, respectively.

Discussion

Amylose becomes radioactive before amylopectin in wheat starch from plants given carbon-14. This is true regardless of the time of day that wheat plants in the early dough stage are injected with sucrose-C^{14}. This same sequence of events occurs when wheat plants in the late dough stage are exposed to $C^{14}O_2$. An unequivocal conclusion is that the path to amylose is more direct than the path to amylopectin. Conversion of amylose to amylopectin by the action of Q-enzyme, isolated from starch-forming tissues of plants, has been repeatedly demonstrated *in vitro*. This mechanism obviously provides a more direct path for amylose formation than for amylopectin formation. However, the data presented here do not eliminate the possibility of a different mechanism.

The gradual decrease in ratio of specific activity of amylose to amylopectin from 56 hours after injection to maturity is about the amount that can be expected from the increase in amylose content of the starch as the wheat approaches maturity (3). This suggests that amylose is not extensively converted to amylopectin, once it has become incorporated into the granule. No evidence is available to suggest that amylose is not converted to amylopectin while these polysac-

charides are being formed but before they are deposited in the granule.

The diurnal variation in rate of incorporation of carbon-14 into wheat starch following stem injections with sucrose-C^{14} can be explained by the variation in translocation rate. It is evident from the data that translocation of sucrose to the head drops to an immeasurably small rate between 11 p.m. and 3 a.m. This agrees with the observation (13) that translocation of sugars to the cotton boll is approximately four times greater during the day than during the night.

Amylose content is not affected by day-length. This seems to indicate that the rate of formation of amylose with respect to amylopectin is the same at night as during the day.

Acknowledgment

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SOME OBSERVED SECONDARY EFFECTS OF HIGH-AMYLOSE GENES IN MAIZE[1]

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ABSTRACT

In the material studied, in which the *ae* and *du* genes were each from a single source, generally as amylose content increased, protein content of the methanol-extracted (fat-free) endosperm increased. Likewise, as amylose increased, kernel weight decreased. When the high-amylose starch genes *ae* and *du* were considered individually, there was a high positive correlation between protein and amylose for the *ae* gene; whereas the *du* gene gave a significant negative correlation. Analysis of the phenotypes suspected of being the double recessive *ae du* indicated there was a negative relationship still between protein and amylose but not so large as for the *du* gene alone. There appeared to be a greater decrease in kernel weight as amylose increased for the *du* gene than for the *ae* gene. However, the possibility of developing high-amylose strains with a relatively low endosperm protein appears promising and worthy of special attention by breeders developing hybrids with high-amylose starch. Such hybrids would be especially beneficial to the corn wet-milling industry where current studies have shown considerable difficulty in the separation of gluten and starch from corn containing more than 50% amylose. *Ae* and *du* genes in other genetic backgrounds might give results different from those reported in this study.

The recent interest in breeding high-amylose strains of maize has given added impetus to investigating the secondary effects of high-amylose genes. If such associations were definitely established, breeding procedures might necessarily require modification. Some of the characteristics that may be associated with high-amylose strains are weight, hardness, and brittleness of the kernel and protein content of the endosperm.

The processing of high-amylose corn by the corn wet-milling method at the Northern Utilization Research and Development Division (1) has indicated difficulty in separating protein and starch. The trouble could be the result of close association between high protein and high amylose. If it were possible to develop a high-amylose corn with reasonably low endosperm protein, the task of separating gluten from starch might lessen.

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Materials and Methods

The assumed double recessive combination of the high-amylose genes *ae* (ha_1) (47–48%) and *du* (ha_2) (35–40%) has given F_3 progeny of 70% (6), and several F_5 progeny with 80% amylose. The *ae* source used in this study was from a stock obtained from Kramer in 1955, with the tentative designation of ha_1, which is now known to be the original *ae* of Bear (4); and the *du* source was from selfed progeny of the Cassel open-pollinated variety with the tentative designation of ha_2. Recently, it was proposed by Kramer *et al.* (4) that ha_1 and ha_2 be designated as *ae* and *du*, respectively. The general procedure followed at the Missouri Agricultural Experiment Station has been to convert standard inbreds separately to *ae* and *du*. After an inbred line has been converted to these two genes through adequate backcrossing, the conversions are then crossed and selfed to recover the double recessive.

Currently, numerous inbreds are being converted to *ae* and *du*. The breeding procedure in making the conversions is to self plants in the segregating progeny and backcross these plants to the recurrent inbred parent. The selfed progeny gives segregations of normal and tarnished kernels. The tarnished kernels are analyzed for amylose content, and ears with the highest amylose content are identified. Since these ears are from plants that were backcrossed to the recurrent inbred parent, this backcrossed progeny is planted in the following generation. The procedure is to be repeated until an inbred has been backcrossed five or more generations.

The results from this breeding operation afforded an opportunity to observe some of the secondary effects of the high-amylose genes *ae* and *du*. The amylose content was determined by potentiometric titration with iodine. Endosperm was separated by hand, ground to pass a 40-mesh screen, and extracted for 24 hours with 85% methanol to remove oil which interferes with the amylose determination. Some nitrogenous materials were removed during the methanol extraction. The methanol-extracted (fat-free) endosperm was washed with distilled water, dried, and reground. Moisture was determined by drying 5 hours in a vacuum oven at 105°C. Nitrogen was determined by the Kjeldahl-Wilfarth-Gunning method (2) with collection of the sample in boric acid as originally done by Wagner (5). The weight of endosperm taken for titration to determine amylose content of the starch was corrected for moisture and protein contents; the amount of fiber present was ignored, since it has been found to be small (not over 5%) and relatively constant. The amylose determinations were made as previously described (3).

The protein content ($N \times 6.25$) referred to throughout this paper

is that of the methanol-extracted (fat-free) endosperm. Part of the protein had been removed by the extraction.

Results and Discussion

Observations made from conversions to *ae* and *du* in 1956 are reported in Table I and summarized in Table II. Each comparison of normal and tarnished kernels in Table I is on an individual ear basis. Although the number of observations was limited, these are believed

TABLE I

COMPARATIVE KERNEL WEIGHT, PERCENTAGE PROTEIN,[a] AND PERCENTAGE AMYLOSE FOR NORMAL AND TARNISHED KERNELS SELECTED FROM SEGREGATING EARS BEING CONVERTED TO EITHER *ae* OR *du* (1956 DATA)

CULTURE No.	NORMAL KERNELS			TARNISHED KERNELS		
	Average Weight	Protein	Amylose	Average Weight	Protein	Amylose
	g	%	%	*g*	%	%
	ae Conversions					
44–1	0.289	7.88	25.0	0.257	9.25	47.2
44–5	.202	7.69	26.8	.180	9.19	45.4
45–13	.293	8.13	25.7	.276	9.31	47.0
46–2	.283	7.00	30.0	.259	7.69	41.1
73–11	.242	9.19	26.8	.215	10.44	41.3
74–9	.196	7.31	32.0	.177	8.56	46.0
76–2	.246	8.06	27.0	.213	9.13	50.0
77–5	.265	8.25	27.7	.222	9.94	48.8
78–6	.242	8.00	26.7	.211	8.94	45.8
83–4	.269	8.19	25.9	.243	9.88	51.3
99–2	.238	9.50	29.5	.213	11.38	45.8
101–4	.251	7.75	30.5	.226	8.19	45.8
104–10	.201	9.00	25.6	.179	10.06	50.2
112–6	.248	10.06	25.7	.217	11.63	60.5
117–3	.248	7.56	26.6	.211	9.06	53.7
187–6	.192	7.88	26.4	.168	9.94	47.7
189–9	0.236	8.56	28.1	0.204	10.25	49.4
Mean	0.244	8.24	27.4	0.216	9.59	48.1
	du Conversions					
38–8	0.228	6.63	27.3	0.202	7.00	33.7
39–3	.213	9.00	26.9	.193	9.88	34.2
58–7	.210	9.56	28.3	.193	10.81	34.4
62–3	.251	8.00	31.8	.227	8.63	32.3
66–7	.304	8.19	25.9	.295	9.69	31.2
68–6	.292	8.81	26.4	.273	9.69	32.6
124–4	.255	9.63	27.7	.242	9.56	34.2
128–4	.236	10.50	25.6	.199	10.25	35.6
135–1	.265	8.38	29.6	.240	7.44	35.0
136–8	.273	7.94	27.0	.242	8.88	35.4
139–6	.275	7.25	26.1	.244	7.50	33.1
142–2	0.298	6.38	26.6	0.267	6.69	32.9
Mean	0.258	8.36	27.4	0.235	8.84	33.7

[a] Protein in endosperm after extraction with 85% methanol.

to be sufficient to indicate a trend in the association of average kernel weight and protein percentage of the methanol-extracted (fat-free) endosperm with amylose content for normal and tarnished kernels, if it is assumed that a significant positive correlation exists between protein of the methanol-extracted (fat-free) endosperm and protein content of the endosperm without extraction.

The mean difference for average kernel weight shows that the normal kernels were significantly heavier than the tarnished among the *ae* conversions; whereas the difference was not significant for the *du* conversions even though the normal kernels were generally heavier. The protein content in the methanol-extracted endosperm was higher for the tarnished selections, especially for the *ae* conversions. The mean difference between normal and tarnished kernels for amylose content was significant for both *ae* and *du* conversions.

TABLE II

MEAN DIFFERENCES FOR AVERAGE KERNEL WEIGHT, PROTEIN,[a] AND AMYLOSE PERCENTAGES OF NORMAL AND TARNISHED SELECTIONS FOR *ae* AND *du* CONVERSIONS (1956 DATA)

	AVERAGE KERNEL WEIGHT		PROTEIN		AMYLOSE	
	ae	*du*	*ae*	*du*	*ae*	*du*
			%	%	%	%
Normal kernels	0.244	0.258	8.24	8.36	27.4	27.4
Tarnished kernels	0.216	0.235	9.59	8.84	48.1	33.7
Difference	0.028*	0.023	1.35**	0.48	20.7**	6.3**

[a] Protein in endosperm after extraction with 85% methanol.

The correlation coefficients given in Table III between kernel weight and protein indicate no relationship for either *ae* or *du* conversions. Kernel weight appears to be negatively associated with amylose content among the tarnished kernels for the *du* conversions and percent protein is positively associated with the amylose content of the tarnished kernels of the *ae* conversions.

TABLE III

CORRELATION COEFFICIENTS FOR AVERAGE KERNEL WEIGHT VS. PROTEIN PERCENTAGE,[a] AVERAGE KERNEL WEIGHT VS. AMYLOSE PERCENTAGE, AND PROTEIN PERCENTAGE VS. AMYLOSE PERCENTAGE FOR NORMAL AND TARNISHED SELECTED KERNELS FROM *ae* AND *du* CONVERSIONS (1956 DATA)

COMPARISON	CORRELATION COEFFICIENTS			
	Normal Kernels		Tarnished Kernels	
	ae	*du*	*ae*	*du*
Average kernel weight vs. percent protein	−0.06	−0.42	−0.20	−0.23
Average kernel weight vs. percent amylose	−0.19	−0.25	0.08	−0.63*
Percent protein vs. percent amylose	−0.34	−0.05	0.48*	0.15

[a] Protein in endosperm after extraction with 85% methanol.

The 1956 results indicate special attention should be given to selecting for low protein in *ae* conversions and for a high kernel weight in the *du* conversions. It is assumed that a low kernel weight would adversely affect the yield of a high-amylose hybrid.

The influence of these various characteristics in *ae du* strains is of special interest. A limited number of comparisons were made in 1957 between protein content and amylose percentage. These results are summarized in Table IV.

TABLE IV

MEAN PROTEIN[a] AND AMYLOSE PERCENTAGE, AND THE REGRESSION AND CORRELATION COEFFICIENTS BETWEEN PROTEIN AND AMYLOSE FOR EARS REPRESENTING VARIOUS RANGES IN AMYLOSE CONTENT (1957 DATA)

AMYLOSE RANGE	NO. OF EARS	MEAN		PROBABLE GENOTYPES	COEFFICIENT	
		Protein	Amylose		Correlation	Regression
%	*No.*	%	%			
21–30	65	9.38 ± 1.65	27.1 ± 2.55	*Ae Ae Du Du*	–0.103	–0.159
31–40	197	9.71 ± 1.36	36.0 ± 2.40	*Ae Ae du du*	–0.599**	–1.054
41–50	131	9.82 ± 1.13	46.2 ± 2.98	*ae ae Du Du*	0.197*	0.522
51–60	118	10.82 ± 0.37	55.6 ± 2.90	*ae ae Du Du*	0.481**	3.735
61–70	81	11.19 ± 1.38	65.2 ± 2.78	*ae ae du du*	–0.362**	–0.729
71–80	7	11.28 ± 1.17	74.0 ± 2.34	*ae ae du du*	–0.373	–0.748
Total	599					

[a] Protein in endosperm after extraction with 85% methanol.

A total of 599 ears was classified into six groups on the basis of amylose content. The mean protein and amylose percentages and standard errors of the means were computed for each of these six groups. The probable genotypes are given for each group. However, the genotype for each ear cannot be positively established without backcrossing to the *ae* and *du* stock. In most instances, the single recessive genotype of *ae* or *du* is known, as these ears are from inbreds being converted to either one of these two genes. Most of the doubtful genotypic classifications are those in the 61–70 and 71–80 percentage groups.

When the six groups were considered individually, the mean protein and mean amylose content increased simultaneously. Although this is an important aspect, a matter of more concern is whether it is possible to select strains with high amylose and low protein content within the six groups. To obtain information on this phase, the regression and correlation coefficients between protein and amylose percentages were computed. Within the amylose ranges, the results show that, whenever the single recessive gene *ae* is predominant, a significant positive correlation between protein and amylose exists, but when *du* is predominant, a significant negative correlation occurs. These results for

ae corroborate the correlation coefficients obtained between protein and amylose percentages in the 1956 study. A negative relationship occurred between protein and amylose for the 61–70% and 71–80% amylose groups where the majority of genotypes are assumed to be the double recessive, *ae du*.

The results of these studies indicate that the gene *ae* from the single source used increases amylose and protein simultaneously with decreased kernel weight but that the *du* gene has a less pronounced effect on the relationship between protein and amylose. It is concluded from these studies that it would be more difficult to select a high-amylose strain with a low protein among the *ae* conversions. However, when the *du* gene used is added to the genotype to give the double recessive *ae du*, the likelihood of selecting a high-amylose strain with a low protein is increased.

It appears that the protein content of the endosperm will be higher and the kernel weight will be lower in high-amylose corn than in normal dent corn, regardless of whether *ae*, *du* (from the sources studied), or the combination is present in a given genotype. Therefore, breeders of high-amylose strains of corn should give special attention to protein content of the endosperm as well as to its amylose content.

Preliminary findings at the Missouri Agricultural Experiment Station suggest that under adverse growing conditions amylose content decreases and protein of the methanol-extracted (fat-free) endosperm increases. Therefore, the genetic-environmental interaction also must be taken into consideration in the breeding of high-amylose strains with a low endosperm protein.

Since the *ae* and *du* genes used in this study each trace to a single source, it is possible that different sources for these two genes might give results differing from those reported in this paper.

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A RAPID METHOD FOR DETERMINING ALPHA-AMYLASE ACTIVITY[1]

SVEN HAGBERG[2]

ABSTRACT

A simple and rapid method for determination of amylase activity of flour and pulverized grain is described. The test may be completed in one or two minutes, depending on the amount of alpha-amylase present.

The principle of the method depends on heating a flour-water suspension of 5 g. flour in 25 ml. water very rapidly to determine the time required to gelatinize and, subsequently, to liquefy the flour suspension. For practical purposes the "liquefaction time" is usually sufficient for evaluating the diastatic properties. However, from the "liquefaction time" and the "gelatinization time," both of which may be determined in the same test, the "diastatic number" may be computed. The "diastatic numbers" for different flours are related linearly to the alpha-amylase activity over a broad range.

The use of variable alpha-amylase concentration to modify fermentation and dough properties and the quality of the baked products is a well-established practice. In order to measure and control alpha-amylase, numerous methods have been developed. These methods involve measurement of sugar or fermentable carbohydrate as affected by the alpha-amylase concentration, extraction of the alpha-amylase and determination of its concentration by its effect on a suitable substrate, and measurement of the effect of alpha-amylase on a starch substrate as it becomes available through gelatinization. Methods for determination of alpha-amylase effects have been reviewed by numerous investigators (1,2,3,4,5,6,7).

It is desirable in many instances to have methods for determination of the effect of alpha-amylase that are rapid, simple, nonexpensive, and, at the same time, accurate. It is convenient if the values obtained have a linear relationship to the concentration of the alpha-amylase. This is particularly important when testing sprout-damaged grain or flour. The method must be sensitive enough to measure alpha-amylase of flours or grain with low activity.

Some of the well-known methods require costly apparatus, are frequently too time-consuming, or do not give a true reflection of the enzyme concentration as related to dough performance or bread characteristics. Among methods which do not require expensive apparatus are those of Sandstedt, Kneen, and Blish (11) and Ritter (10). These methods, however, may be quite time-consuming, particularly if

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only a few samples are to be tested simultaneously. These methods also have the common fault that they measure time required to cleave starch to a much greater degree than actually occurs during bread-baking. In the modified Wohlgemuth procedures (10, 11), starch cleavage proceeds until units of about 7 glucose units remain. Myrbäck and Gjörling (8) have pointed out that viscosity of the gelatinized starch drops 50% when only 0.1% of the glucosidic linkages are opened; whereas 7% of the linkages must be opened before the remaining dextrins fail to give a blue color with iodine (9). It is evident in actual baking that dextrinization and saccharification proceed much more slowly than liquefaction and that the point never is reached in bread during baking when starch is split to products not deeply colored by iodine.

It is recognized that liquefaction of a viscous gelatinized starch paste can be followed by the common viscometric procedures. Not only are the apparatus for this expensive, but they fail to give results which are related linearly to the alpha-amylase concentration over a broad range. The lack of linearity appears to be due to the fact that the temperature is raised too slowly for samples having high alpha-amylase concentration. In these cases, an appreciable decrease in the substrate concentration occurs.

The object of this research has been to develop a simple, rapid method of assay of alpha-amylase activity, which will give results that are linear over a wide range of alpha-amylase concentrations. The method is based upon rapid gelatinization of the substrate and subsequent measurement of the liquefaction time.

Description of the Method

Apparatus (1) Water bath (100°C.). The thermostatically controlled, 600-watt bath, equipped with test tube rack, approximately 20 cm. high by 15 cm. diameter (Fig. 1).

(2) Test tubes, inner diameter 21 mm., length 220 mm.

(3) Stop watch. An electric flash watch adjustable to 1 second is desirable (Fig. 1).

(4) Stirrer-viscometer. Weight 25 g. (Fig. 1). This stirrer is equipped with two stop-gages separated by 80 mm. A mechanical stirrer can be attached if desired.

Determination: Five grams of flour or finely pulverized grain and 25 ml. distilled water (20°C.) are thoroughly shaken by hand in the test tube using 20 vigorous shakes. The test tube with the stirrer-viscometer is immersed in the boiling-water bath and the stop watch started. The surface of the suspension in the test tube should be at least 5 cm. under the surface of the boiling water. Five seconds after immersion of the test tube, stirring of the suspension is started by movement of the stirrer up and down 2 times per second.

Two basic measurements based on change of viscosity of the suspension are recorded. "Gelatinization time" is defined as the time of heating required to develop a paste in which the stirrer-viscometer drops by its own weight a distance of 70 mm. in 0.25 second. Gelatinization time will vary from 25 to

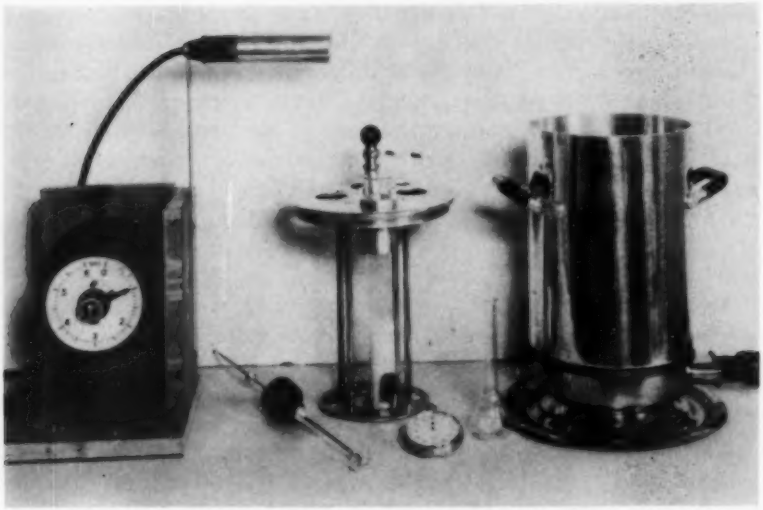

Fig. 1. An apparatus for determination of gelatinization time, liquefaction time, and diastatic number of flour. Right: water bath. Middle: test tube rack and stirrer-viscometer. Left: electrical flash watch, adjustable to 1 second.

40 seconds for wheat flour and from 20 to 35 seconds for rye flour. After the gelatinization time has passed, the paste will rapidly become thicker. The second measurement, "liquefaction time," is defined as the total time of heating required to reduce the thick paste into a thin paste in which the stirrer-viscometer will drop a distance of 70 mm. in 1 second by its own weight. Liquefaction time will vary from 30 seconds or less for wheat and rye flours with high amylase activity (e.g., flour from sprout-damaged grain) up to 300 seconds or more for flour with low amylase activity.

Calculation of the "Diastatic Number." The "diastatic number" may be calculated from gelatinization time and liquefaction time, assuming a diastatic index of 100 when 100% of the starch is liquefied in 60 seconds.

$$\text{diastatic number} = \frac{100 \times 60}{\text{liquefaction time} - \text{gelatinization time}}$$

Discussion

The method described has been found to give reliable results over a wide range of alpha-amylase concentrations. The relationships between several measurements of alpha-amylase activity and the amount of sprouted Swedish winter wheat flour present in a mixture of Manitoba wheat flour with low amylase activity are shown in Fig. 2. These data demonstrate the linear relationship between diastatic number, SKB alpha-amylase units, and the amount of alpha-amylase added to a flour. The relationship is linear over the range usually encountered.

This is in contrast to viscometric methods that depend on relatively slow heating of the suspension. The diastatic number expression will range from 20 or less to over 600 for the usual range of alpha-amylase activities of flours or wheats of commerce. This wide range of values together with the precision with which the numbers can be determined cause the method to be of value in the laboratory where a rapid, inexpensive, and sensitive method is required.

The rate of heating the suspension is important. If the method is followed as outlined, the temperature can be expected to rise as follows:

After 1 minute in boiling water – 77°C.
After 2 minutes in boiling water – 90°C.
After 3 minutes in boiling water – 95°C.

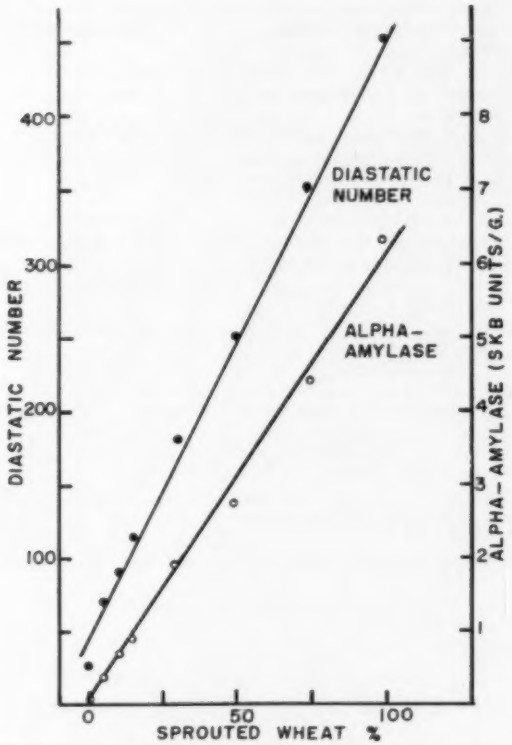

Fig. 2. Relationship between diastatic number, alpha-amylase, and the amount of sprouted Swedish grain in mixture.

If a water bath of lower temperature is employed, the temperature must be high enough so that the starch is gelatinized faster than it is cleaved by the enzyme.

The ratio of flour to water may be varied. Thus, for low diastatic flours, less than 5.0 g. may be employed, and, if high diastatic flours are common, the amount of flour may be standardized at a higher level than 5.0 g. per 25 ml. of water.

For practical purposes, it is usually sufficient to simply determine the liquefaction time according to the method given.

Acknowledgment

The author is indebted to John A. Johnson, Kansas State University, Manhattan, Kansas, for assistance in the preparation of this manuscript.

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TREATMENT OF WHEAT WITH IONIZING RADIATIONS
V. Effect of Gamma Radiation on Some Enzyme Systems[1]

PEKKA LINKO AND MAX MILNER[2]

ABSTRACT

Germination (wet filter paper), dehydrogenase (as indicated by TTC reduction), glutamic and pyruvic acid decarboxylase, alpha-amylase, and protease activities and maltose value, were determined in wheat samples after treatment with gamma radiation at levels of 0.05, 0.1, 0.5, 1.0, and 3.0 megarep. Germination was reduced even by the lowest radiation treatment (0.05 megarep), whereas the TTC test with whole kernels indicated no significant decrease before the 1.0 megarep treatment. Spectrophotometric determination of formazan produced from TTC by ground grains showed a regular but curvilinear decrease in relation to radiation applied, considerable formazan color remaining even in completely nonviable grain treated with 3.0 megarep. These data suggest that at least a part of the reduction of TTC to formazan is caused by irradiation-produced factors; browning reaction products are the most probable.

Glutamic and pyruvic acid decarboxylase activities both decreased to about half of the original value in grain treated with 3.0 megarep.

Maltose value showed a considerable rise with radiation level due to increased starch susceptibility, whereas alpha-amylase activitity was virtually unchanged even after 3.0 megarep. There was similarly no impairment of protease activity.

In the course of a study of the effects of gamma radiation on the respiratory, biochemical, and technological properties of wheat, it was noticed (13) that dosages between 125,000 and 625,000 rep eliminate fungal respiration in the grain, apparently without increasing its protein solubility or fluoresence value. The effect of irradiation on the enzymes of the grain itself becomes important in any evaluation of irradiation as a practical way to sterilize grain. Holmes (5) has recently written an extensive review of the biochemical effects of ionizing radiations, including the effects on various enzymes other than those in wheat. The purpose of the present work was to investigate the effects of gamma-rays on certain enzyme systems in wheat grains.

Materials and Methods

Material. Wheat used in this study was a composite of several standard hard red winter varieties of good breadmaking quality, produced at various experimental fields throughout the U.S. hard red winter

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[2] Respectively, Assistant Professor and Professor, Kansas State University.

wheat area in 1957 and 1958. The composite sample contained 13.4% protein (14% moisture basis) and 1.6% ash. Germination, as estimated with moist filter paper in Petri dishes, was 94–96%. The moisture content, as determined by drying ground (2 minutes by Waring Blendor) material 1 hour at 130°C. in a forced-draft air oven, was 9.0% (wet-weight basis). The grains used for enzyme experiments were ground by micro-Wiley mill to pass a 20-mesh sieve.

These wheats were sealed in No. 2 metal cans and sent to the Materials Testing Reactor of the U.S. Atomic Energy Commission, Idaho Falls, Idaho, for radiation treatment. Radiation dosages of 0.0, 0.05, 0.1, 0.5, 1.0, and 3.0 megarep were used. After their return, the irradiated cans were opened and each treatment group was mixed by a MacLellan batch mixer for 30 minutes prior to sampling for enzyme activity determinations.

Germination Test. One hundred kernels were surface-sterilized by soaking 2 minutes in 0.1% mercuric chloride solution, followed by a thorough rinsing in tapwater. The grains were then allowed to germinate between moist filter papers for 7 days at 25°C. Germinated grains were counted and removed every second day.

Dehydrogenases. Dehydrogenase activity was determined by two different methods. In the first method kernels were soaked overnight in distilled water, cut lengthwise, and again soaked overnight in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC). The percentage of colored embryos was then estimated by visual examination.

The second method was a modification of that proposed by Sorger-Domenigg *et al.* (12). Five milliliters of 1% TTC solution buffered to pH 7.3 with 0.067*M* phosphate buffer were added to a test tube containing 1 g. of ground grains. After two vacuum infiltrations, the test tubes were incubated 1 hour at 38°C. Formazan produced was extracted with 25 ml. of acetone, and the transmittance was measured at 520 $m\mu$ with a Beckman Model DU spectrophotometer.

Decarboxylases. Glutamic and pyruvic acid decarboxylase activities were determined manometrically as previously described by Cheng *et al.* (2,3). The activities were reported as microliters of carbon dioxide produced during 30 minutes from 1 ml. of 0.1*M* glutamate (pH 5.8) by 500 mg. of ground grain.

Alpha-Amylase. Alpha-amylase activity measurements were based on methods developed by Sandstedt *et al.* (11) and Redfern (10). The procedure was modified slightly because of the low enzyme activities encountered. Ground grains (2.5 g.) were extracted with 50 ml. of 0.2% calcium chloride solution. Ten milliliters of this solution were used without further dilution for the alpha-amylase determination. Twenty

milliliters of soluble starch substrate solution were used (equivalent of 0.01 g. of starch).

Maltose Value. Maltose value was determined according to the procedure described in *Cereal Laboratory Methods* (1).

Protease. Protease activity was determined according to Miller and Johnson (8), except that 1.3334 g. of ground kernels were used, and the final results were accordingly multiplied by 3.

Results and Discussion

Figure 1 shows that results of viability determinations vary greatly with the method used. The ordinary germination test between filter papers indicates viability to decrease rapidly with increasing radiation dosage; 0.5 megarep retards root growth completely. However, when kernels cut lengthwise were soaked in TTC solution, the coloring of the embryos remained virtually the same up to 0.5 megarep dosage. The

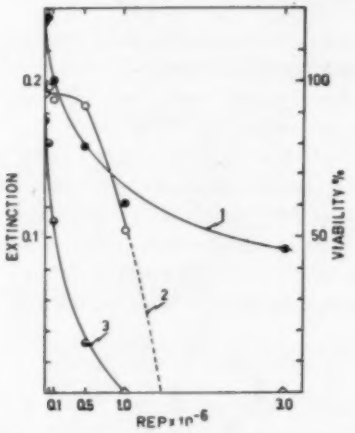

Fig. 1. The effect of gamma-irradiation of wheat grains on viability. 1: 2,3,5-triphenyltetrazolium chloride test with ground grains; 2: the same with lengthwise-cut kernels (visual examination); 3: germination test. (Curve 1 is related to extinction scale; 2 and 3 to the viability percent scale.)

first significant decrease in viability as observed by a color change occurred at 1.0-megarep dosage. No coloring remained after 3.0-megarep irradiation. On the other hand, when dehydrogenase activity was determined spectrophotometrically in ground material, it was found that even after irradiation treatment at 3.0 megarep the formazan color intensity decreased only about 60% from the control. This result might suggest that dehydrogenase activity, which produces formazan, is rela-

tively resistant to radiation. In general, many enzymes have been found to be quite radioresistant. Thus, Green (4), for example, found that 2.0 megarep, which was sufficient to kill microorganisms, caused only a 29% reduction in the proteolytic activity of calf heart. In the case of TTC, however, it is possible that the reducing compounds which may be formed during irradiation can nonenzymatically reduce TTC to formazan. Thus, if prior to the TTC treatment the enzymes of the ground material were inactivated by heating for 15 minutes at 150°C., the formazan color intensity increased with increasing radiation from almost nil approximately to the same level found with normal determination at 3.0 megarep dosage. Since high radiation levels stimulate a Maillard reaction in wheat (9,13), it is probable that reductones formed during the browning reaction may partially account for nonenzymatic production of formazan from TTC.

Figure 2 shows that 3.0-megarep irradiation of wheat kernels causes approximately 50% decrease in glutamic acid and pyruvic acid decarboxylase activities.

Alpha-amylase activity is normally very low in intact wheat grains (11). Table I shows that alpha-amylase activity remains virtually unaffected by irradiation up to 3.0 megarep, as is also the case with protease activity. Maltose value increases greatly with increasing radiation dosage, which agrees well with earlier results (7,9). Lee (7) found no increase in the saccharifying power in papain extract of flour from irradiation, suggesting that no actual activation of beta-amylase takes place. Hence, the increase in maltose value, as indicated in previous publications of this series, is due to a sharp increase in the susceptibility of the starch fraction to beta-amylase attack.

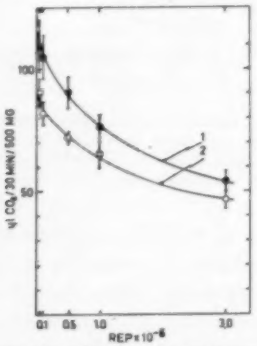

Fig. 2. The effect of gamma-irradiation of wheat grains on 1) glutamic acid decarboxylase, and 2) pryruvic acid decarboxylase activities. The stems on the points indicate standard deviations with each sample as calculated from five replicate determinations.

TABLE I

THE EFFECT OF GAMMA-IRRADIATION OF WHEAT GRAINS ON ALPHA-AMYLASE AND PROTEASE ACTIVITIES, AND ON MALTOSE VALUE

IRRADIATION DOSAGE	ALPHA-AMYLASE ACTIVITY	PROTEASE ACTIVITY	MALTOSE VALUE
megareps	*SKB-units/g*	*Hb-units*	*maltose/10 g*
0.00	0.027	12.4	153
0.05	0.028	12.4	156
0.10	0.026	12.4	179
0.50	0.026	12.2	216
1.0	0.024	12.1	244
3.0	0.020	11.9	315

All these results indicate that high irradiation dosages damage several enzyme systems considerably. However, at dosages sufficient only to kill insects in grain (about 0.05 megarep), the damage may not be great although seed viability will be affected. The long-term effects of deinfestation dosage levels on the technological properties of the grain as affected by storage remain to be determined.

Acknowledgment

The authors are greatly indebted to James R. Fleming for amylase and protease determinations.

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STUDIES WITH RADIOACTIVE TRACERS
IV. Degrees of Reduction of Br^{82}-Labeled Bromate to Bromide by Some Components of Flour[1]

C. C. Lee and R. Tkachuk[2]

ABSTRACT

The treatment of dioxane solutions of fat from petroleum ether extraction of 100 g. of patent or clear flour with aqueous solutions of 1.5 mg. of Br^{82}-labeled potassium bromate at neutral pH or buffered to pH 4.7, in all cases did not give any measurable conversion of bromate to bromide after such mixtures were shaken for 4 hours. Similar treatment also caused no significant change in the fatty acid composition of the petroleum ether-extracted fat from patent or clear flour as measured by gas-liquid chromatography. Such results appear to indicate the absence of direct reactions between bromate and the petroleum ether-extracted fat, at least when treated in dioxane-water mixture. The previously reported pronounced effects of defatting on the degree of decomposition of bromate to bromide by water-flour doughs may possibly involve factors in the dough system besides the bromate and the fat. Water and flour slurry, water and freeze-dried gluten slurry, and the aqueous extract of flour, all can reduce some bromate to bromide. The results support the conclusion that bromate, a nonspecific oxidizing agent, may take part in reactions with a number of constitutents of flour.

In paper III of this series (6) it was reported that defatting of flours by extraction with petroleum ether markedly decreased the degree of reduction of bromate to bromide by water-flour doughs made from commercially milled, patent, intermediate and clear flours initially treated with 15 p.p.m. Br^{82}-labeled potassium bromate. The data indicated that the petroleum ether-extractable components of flour were involved, either directly or indirectly, in the decomposition of bromate by water-flour doughs. The present work includes attempts to ascertain whether the petroleum ether extract of flour can directly reduce bromate to bromide. The degrees of reduction of bromate to bromide by water slurries of flour and of freeze-dried gluten, and by aqueous extracts of flour, were also measured.

Materials and Methods

Untreated, patent and clear flours, commercially milled from Western Canadian hard red spring wheat, were used in these experiments. The contents of protein, ash, and petroleum ether-extracted fat in

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[2] Present address: Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Manitoba.

these flours, on a 14% moisture basis, are listed below:

	Protein (N × 5.7)	*Ash*	*Fat*
	%	%	%
Patent	12.8	0.35	0.79
Clear	16.8	0.80	1.80

The fat, obtained from petroleum ether extraction of 100 g. of flour in a series of Goldfisch fat extractors, was taken up in 15 ml. of purified dioxane. A solution of 1.5 mg. (15 p.p.m. based on the 100 g. of flour) of Br^{82}-labeled potassium bromate (7) in 15 ml. of distilled water was added. The mixture was shaken mechanically for 4 hours. Aliquots were withdrawn for the determination of any reduction to bromide by a method involving fractional precipitation of silver bromide described previously (7). Duplicate runs were carried out with fats from the patent and clear flours. Since aqueous extracts of water-flour doughs are slightly acidic, possible effects of acidity were also investigated by shaking the dioxane solution of fat with 1.5 mg. of labeled potassium bromate in 15 ml. of acetic acid-sodium acetate-buffered solution of pH 4.7.

The fatty acid composition in the petroleum ether-extracted fat, either as such or after shaking with 1.5 mg. of potassium bromate in dioxane-water for 4 hours and then re-extracted with petroleum ether, was analyzed by gas-liquid chromatography.[3] The detailed procedures have been reported elsewhere (3,4).

In the study with flour slurries, 10 g. of patent flour and 100 ml. of distilled water containing 1.5 mg. (150 p.p.m. based on the 10 g. of flour) of Br^{82}-labeled potassium bromate were shaken continuously by a mechanical shaker. At 1, 2, 3, or 4 hours after the beginning of the experiment, portions of the slurry were removed and centrifuged, and the bromide content in the aqueous supernatant determined by the fractional precipitation of silver bromide (7). Similar experiments were carried out with slurries of 1.5 g. freeze-dried gluten and 100 ml. of distilled water containing 1.5 mg. of labeled potassium bromate, or with 1.5 mg. of labeled potassium bromate dissolved in 100 ml. of aqueous extract of flour. The freeze-dried gluten was prepared from the patent flour according to the method of Lusena (8). The aqueous extract of flour used in this experiment was obtained from shaking patent flour with distilled water (100 ml. water per 10 g. flour) for 1 hour, followed by centrifugation.

Results and Discussion

Absence of Bromide in the Treatment of Fat with Bromate in Dioxane-water. The treatment of dioxane solutions of petroleum ether-

[3] Kindly carried out by B. M. Craig of the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Sask.

extracted fat from either patent or clear flour with aqueous solutions of labeled potassium bromate at neutral pH or buffered to pH 4.7, as outlined in the previous section, gave, in all cases, no measurable conversion of the bromate to bromide. A typical set of experimental results is as follows:

A dioxane solution of fat from 100 g. of patent flour was shaken for 4 hours with an aqueous solution of 1.5 mg. of Br^{82}-labeled potassium bromate. The resulting mixture was centrifuged and 0.5 ml. of the dioxane-water layer was evaporated to dryness for counting. This sample represented the total Br^{82} activity.

To 5.0 ml. of the dioxane-water layer was added 1.0 ml. of solution containing 30.00 mg. of ordinary potassium bromide and 30.00 mg. of ordinary potassium bromate as carriers. Another 1.0 ml. of solution containing 42.82 mg. of silver nitrate, the molar equivalent of 30.00 mg. of potassium bromide, was introduced. The precipitate was centrifuged off and 0.5 ml. of the supernatant evaporated to dryness for counting. This sample represented the activity remaining after removal of all the bromide.

The sample representing total activity was counted immediately before and after the counting of the sample containing the activity after bromide removal. By means of this "sandwiching" technique, the activities of the two samples were directly comparable, thus eliminating the necessity of any correction for radioactive decays. The mean value for the two counts of the sample containing total activity was 1805 c.p.m., while the activity of the sample prepared after bromide removal was 1304 c.p.m. Since the precipitation of the silver bromide involved a dilution of the dioxane-water solution from 5.0 to 7.0 ml., taking this into account, the corrected activity in the sample after bromide removal was $1304 \times 7/5 = 1826$ c.p.m., essentially the same as the sample containing total activity, thus indicating no conversion of bromate to bromide.

The fatty acid composition of the petroleum ether-extracted fat, before and after treatment of the fat from 100 g. of flour with 1.5 mg. of potassium bromate in dioxane-water for 4 hours, as determined by gas-liquid chromatography, is given in Table I. The results clearly showed that the bromate treatment in dioxane-water caused no significant changes in the fatty acid composition, which supports the finding that such treatment also caused no reduction of bromate to bromide.

Possible Interaction between Bromate and Fat in the Dough. The results discussed so far indicated that there appears to be no direct reaction between bromate and petroleum ether-extracted fat when shaken in dioxane-water mixture. In the dough, the bromate and fat

TABLE I

FATTY ACID COMPOSITION OF PETROLEUM ETHER-EXTRACTED FAT FROM PATENT AND CLEAR FLOURS BEFORE AND AFTER TREATMENT WITH POTASSIUM BROMATE IN DIOXANE-WATER MIXTURE

FATTY ACID[a]	FAT FROM PATENT FLOUR		FAT FROM CLEAR FLOUR	
	Before Bromate Treatment	After Bromate Treatment	Before Bromate Treatment	After Bromate Treatment
	%	%	%	%
Myristic	0.1	0.1	0.1	0.1
Pentadecanoic	0.1	0.1	0.1	0.1
Palmitic	19.0	18.3	16.7	17.3
Palmitoleic	0.4	0.4	0.4	0.4
Stearic	0.9	1.0	0.9	0.8
Oleic	13.7	14.0	18.2	17.6
Linoleic	61.3	61.6	58.2	58.3
Linolenic	3.2	3.2	3.8	3.6
Eicosenoic	1.3	1.3	1.6	1.8

[a] The presence of minor quantities of less than 0.1% of some fatty acids of lower molecular weight was not accurately measured in the current study.

are present in a much more complex system than in dioxane-water. It has been found that defatting of the flour greatly decreased the amount of bromate reduced to bromide by water-flour doughs (6). This finding, considered together with the absence of any direct reaction between bromate and fat outside of the dough system, pointed to some interaction between bromate and fat in the dough, though such interaction does not have to be a direct reaction but may involve other dough constituents besides the bromate and the fat. From a study on the rates of bromate loss in doughs from defatted and reconstituted flours, Cunningham and Hlynka (5) have reached a similar conclusion that lipid acts as an intermediate between bromate and reducing materials in the flour rather than reacting directly with bromate.

Fatty Acid Composition of the Petroleum Ether Extract of Flour. The quantitative determination of the fatty acid composition of the petroleum ether extract of flour is of interest in itself. Early work by classic methods, such as fractional distillation of the methyl esters and calculation for linoleic and linolenic acids from iodine and thiocyanogen numbers, gave only rough estimations of the most common C_{16} and C_{18} acids (1,9). More recently, Coppock *et al.* (2) reported a qualitative determination of the fatty acid composition of a carbon tetrachloride extract of flour by gas-liquid chromatography. Their findings, indicating that there are large proportions of oleic and linoleic acids relative to the small amount of stearic acid in the C_{18} fraction and that the C_{16} fraction consists mainly of palmitic acid, are in agreement with the present results. These workers (2), however, also reported the presence of a "large unsaturated C_{19} fraction" in their carbon tetrachloride-extracted fat. Such a fraction was not observed in the current studies.

Instead, eicosenoic acid (C_{20}, one double bond) was found to be present in amounts of the order of 1.5% in the petroleum ether-extracted fat of both patent and clear flours.

Reduction of Bromate to Bromide by Water-Flour Slurry, Water-Gluten Slurry, and Aqueous Extract of Flour. The extents of reduction of 1.5 mg. of labeled potassium bromate in slurries of 100 ml. of water and 10 g. of patent flour or of 100 ml. of water and 1.5 g. freeze-dried gluten (1.5 g. is the approximate amount of freeze-dried gluten obtainable from 10 g. of patent flour), as well as the reduction of 1.5 mg. of labeled potassium bromate dissolved in 100 ml. of aqueous extract of patent flour, are given in Table II. Some reduction of bromate to bromide was observed in all samples examined, though the bromide contents in the supernatants from the slurries or in the aqueous extract, expressed on a percentage basis, were relatively low. These low percentages of conversion are to be expected, as the use of 1.5 mg. of bromate per 10 g. of flour is equivalent to a treatment with 150 p.p.m. of bromate. Unfortunately, the specific activity of the Br^{82} available was not sufficiently high to permit the use of, for example, 0.15 mg. of labeled bromate per slurry containing 10 g. of flour. Nevertheless, the results given in Table II do indicate that flour slurry, freeze-dried gluten slurry, and aqueous extract of flour all can reduce some bromate to bromide. The fact that the degrees of reduction of bromate to bromide by the gluten slurry or by the aqueous extract of flour were lower than the corresponding reduction of bromate by the slurry of whole flour (Table II) may also suggest that whole flour contains materials other than gluten or other than the water-extractable components which could cause reduction of bromate to bromide. This is in

TABLE II

BROMIDE CONTENTS IN WATER-FLOUR SLURRY, WATER FREEZE-DRIED GLUTEN SLURRY, AND IN AQUEOUS EXTRACT OF FLOUR AFTER TREATMENT WITH Br^{82}-LABELED POTASSIUM BROMATE[a]

Reaction Time	Flour Slurry		Gluten Slurry		Aqueous Extract
	Recovery of Br^{82} in Supernatant	Bromide Content in Supernatant	Recovery of Br^{82} in Supernatant	Bromide Content in Supernatant	Bromide Content in Solution
hours	%	%	%	%	%
1	92	3	99	2	2
2	92	4	96	1	5
3	88	6	100	3	4
4	92	12	98	4	6

[a] Treatments were 1.5 mg. of labeled potassium bromate with 10 g. flour, 1.5 g. freeze-dried gluten or aqueous extract of 10 g. flour, total volume in each case being 100 ml. All data were calculated from mean activity values of duplicate experiments.

accord with a previous conclusion that bromate, a nonspecific oxidizing agent, may take part in reactions with a number of constituents of flour, rather than with any particular one (6).

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EFFECTS OF ABSORPTION AND TEMPERATURE UPON FLOUR-WATER FARINOGRAMS[1]

E. G. Bayfield[2] and C. D. Stone[2]

ABSTRACT

By the use of the farinograph, studies with flour-water doughs over a range of temperatures between 81.5° and 121°F. (27.5° and 49.4°C.) indicate that, at constant absorption, consistency softens with increase in temperature. With consistency held constant, the absorption decreases as the temperature increases. As the farinograph has been standardized at 30°C., the customary interpretation of farinograms may not be valid when the curves are obtained at temperatures other than normal.

High-temperature dough fermentation systems (100°–104°F. or 37.8°–40°C.) may have considerable advantage over the present (80°–90°F. or 26.7°–32.2°C.) conventional systems. Published information concerning the newer breadmaking processes gives evidence of a tendency toward higher temperatures than those presently used in commercial production. The object of this study was to find what effect

[1] Manuscript received March 2, 1959. Contribution from the Department of Baking Science & Management, Florida State University, Tallahassee, Florida.

[2] Professor and Assistant Professor, Department of Baking Science and Management.

variations in absorption and temperature have on flour-water farinograms.

Numerous earlier workers have contributed to the development of methods for interpreting farinograms of doughs obtained under ordinary temperatures. Swanson (5) discusses the effects of absorption and temperature on flour-water doughs up to 35°C. Markley and Bailey (4) found that a curvilinear relation existed between absorption and time to bring a dough to the point of minimum mobility. When plotting the logarithm of time required to reach the point of minimum mobility against the flour concentration in the dough, they found that this curvilinear relation could be straightened. Stone and Bayfield[3] found that fermenting yeast doughs exhibited the same trends of temperature effect as were observed with flour-water doughs; dough consistency varied inversely with the temperature, other factors being constant. With the use of a constant absorption, bread baked from doughs fermented at 100°F. (37.8°C.) was comparable or superior to bread baked from doughs fermented at 86°F. (30°C.).

Materials and Methods

A nonbromated, nondiastatted, bleached flour, milled from hard red winter wheat was used. Its analysis was approximately 11.40% protein and 0.43% ash. Distilled water was used, and the flour absorption was 57.2% (14% M.B.) when determined in the farinograph at 86°F. (30°C.) with the peak development at 500 Brabender units.

Farinograms (1) were obtained varying the temperature by increments of 7°F. (3.88°C.) between 86° and 121°F. (30° and 49.4°C.). An interval of 4.5°F. (2.57°C.), from 86° down to 81.5°F. (30° to 27.5°C.), was recorded; this was the lowest temperature that could be maintained with the available water cooling system. The highest temperature was 121°F. (49.4°C.) as this approached the upper limit of yeast activity. White (6) has shown that 100° to 104°F. (37.8° to 40°C.) is the maximum temperature range to obtain optimum fermentation. The flour and water were both preconditioned to the temperatures at which the farinograms were recorded before the water was titrated into the flour in the mixing bowl of the farinograph. Constant flour weight was used throughout; water and temperature were the variables in the experiments.

Fifteen-minute farinograms were obtained over the range of temperatures described above, with constant absorption as one variation and "500 consistency" curve peaks the other. The 500 consistency

[3] Stone, C. D. and Bayfield, E. G. A comparative study of several factors influencing the course of fermentation under three systems of bread production. Presented at the 44th annual meeting, Washington, D. C. (1959).

farinograms required recording a number of curves before the exact consistency desired was obtained.

Results

Figures 1 to 4 and Table I record the results of the farinograph studies. When the temperature was increased from 81.5° to 121° F.

TABLE I
MEASUREMENTS TAKEN FROM FARINOGRAMS RECORDED OVER A RANGE OF TEMPERATURES

Temperature	Constant Absorption (57.25%)				Constant Consistency (500 B.U.[a]) Variable Absorption			
	Peak Time	Mixing Time (minutes)			Peak Time	Water[b] Used	Mixing Time (minutes)	
		Peak	7	15			7	15
°F	*minutes*	*Bu*	*Bu*	*Bu*	*minutes*	%	*Bu*	*Bu*
81.5	7	555	555	478	8	59.40	500	440
86.0	7	500	500	445	7	57.25	500	440
93.0	3	455	438	395	2½	55.50	480	425
100.0	2½	412	375	338	2	54.50	445	398
107.0	2	390	325	290	1⅞	53.80	400	358
114.0	1¾	375	285	258	1¾	52.80	375	332
121.0	1⅔	362	265	232	1⅔	52.40	355	318

[a] B.u. — Brabender units.
[b] Percent absorption based on the flour as 100% (14% M.B.).

(27.5° to 49.4°C.), with constant absorption, there was a decrease in height of the curve of 193 Brabender units. The results for the higher temperatures illustrated in Fig. 1 show considerable similarity to curves obtained from certain weak flours or with results of various enzymes on wheat flours at normal temperatures, as shown by Gray (2) and Johnson and Miller (3). They are also in agreement with the discussion by Swanson (5).

When titrations were adjusted to give a peak value of 500 consistency, there was a decrease in absorption, based on flour as 100%, of 7% between the temperatures of 81.5° and 121°F. (27.5° and 49.4°C.). With the use of constant absorption, the farinogram height decreased as the temperature was increased.

When the data in Table I were graphed (Fig. 2), the absorption-temperature curves (measured for 500 consistency) and the consistency-temperature curves (measured at their peaks with constant absorption) were curvilinear. When the curve peaks were measured at 7-minute mixing time with constant absorption and varied temperature, the curves were less curvilinear in nature and became almost a straight

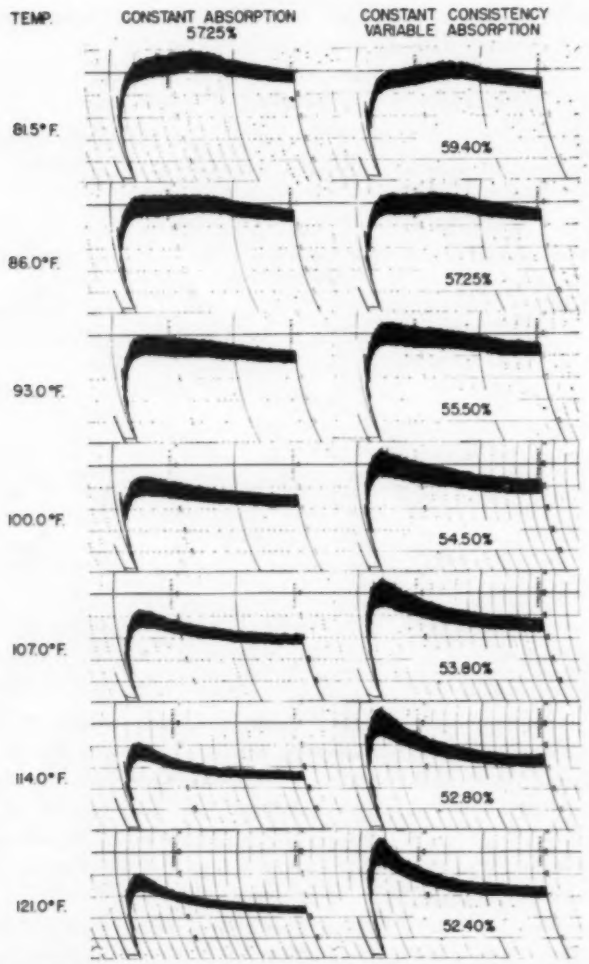

Fig. 1. Effects of varying temperature and absorption upon farinograms.

line between 86° and 107°F. (30° and 41.7°C.).

When these data were plotted on semilogarithmic paper (Fig. 3), the peaks of the constant-consistency and constant-absorption curves approximated a straight line. A logarithmic relation is suggested between flour absorption, mixing time, and dough temperature when one of these three factors is held constant. This is in accord with the work of Markley and Bailey (4) and extends the logarithmic relation

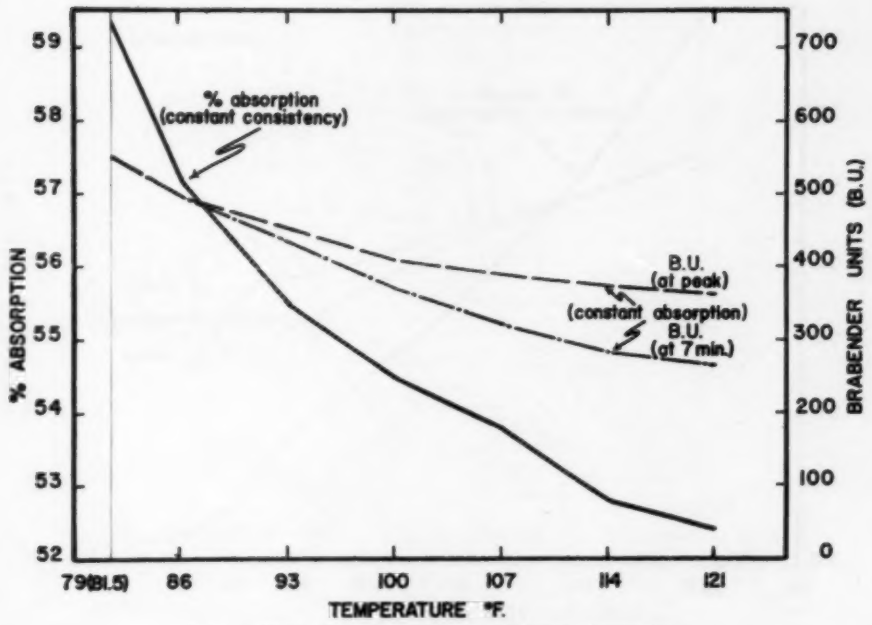

Fig. 2. Effect of temperature upon dough absorption.

over a range of temperatures.

The 7-minute curves were of an inverted curvilinear nature. Measurements at 15 minutes were roughly parallel to the 7-minute curves, but were lower in height (Table I and Fig. 4).

Discussion

This study shows the effects of absorption and temperature upon farinograph flour-water doughs and points out that the farinogram, as now interpreted, does not show correct flour absorption or mixing characteristics when the dough is mixed at higher or lower temperatures than normal.

One may conclude that dough consistency varies inversely with the temperature and, with this particular flour, in a semilogarithmic relationship between the temperatures of 81.5° and 121°F. (27.5° and 49.4°C.). The higher the temperature at which a dough is mixed, the less its absorption at constant consistency, or the softer it becomes at constant absorption.

Studies by Stone and Bayfield (see footnote 3) show that slackness in yeasted doughs of commercial consistency, due to increased tem-

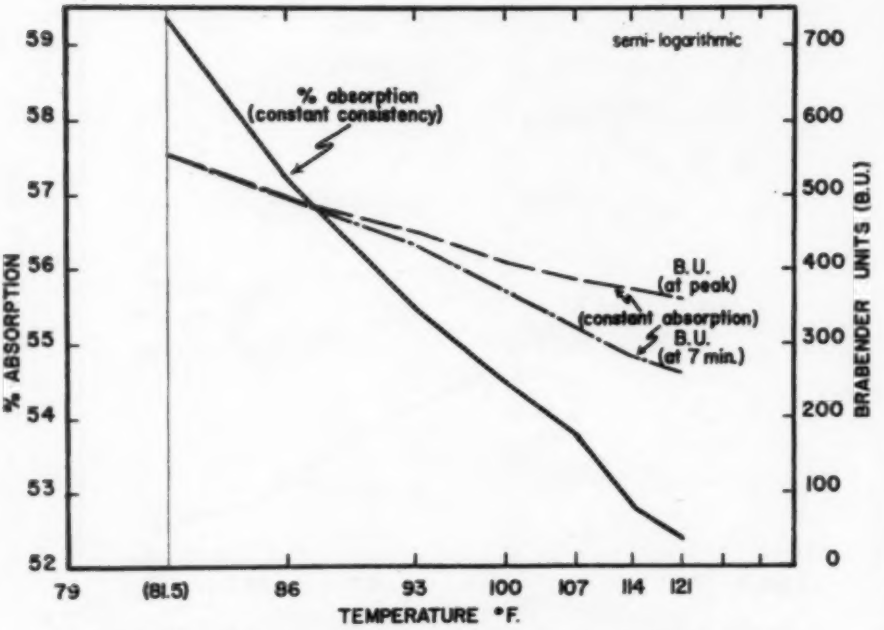

Fig. 3. Effect of temperature upon dough absorption (temperature axis semi-logarithmic).

perature, does not indicate a longer mixing requirement for properly mixed doughs (in terms of finished bread). They found that doughs mixed in the higher temperature range appeared to be too slack, but did not produce baked bread containing excess moisture and did not have excessive moisture loss during proofing and baking.

Data in Table I show the mixing time required to reach the farinogram peaks. Mixing time to the curve peak decreased slowly between 81.5° and 86°F. (27.5° and 30°C.); then it decreased abruptly between 86° and 93°F. (30° and 33.9°C.) and decreased much more slowly at higher temperatures. This indicates that the farinogram peaks as interpreted at 86°F. (30°C.) are not a measure of the same factors as the peaks at the higher temperatures (Fig. 4).

Farinogram interpretations such as optimum mixing time, obtained from normal temperature curves, are not satisfactory at higher or lower temperatures. In Figs. 1 and 4, it appears that the farinogram peaks sink into the curves, rather than shifting to the left (reaching the peak sooner), with increased temperature. This is probably due to decrease in the viscosity of the water films lessening the adhesion tensions

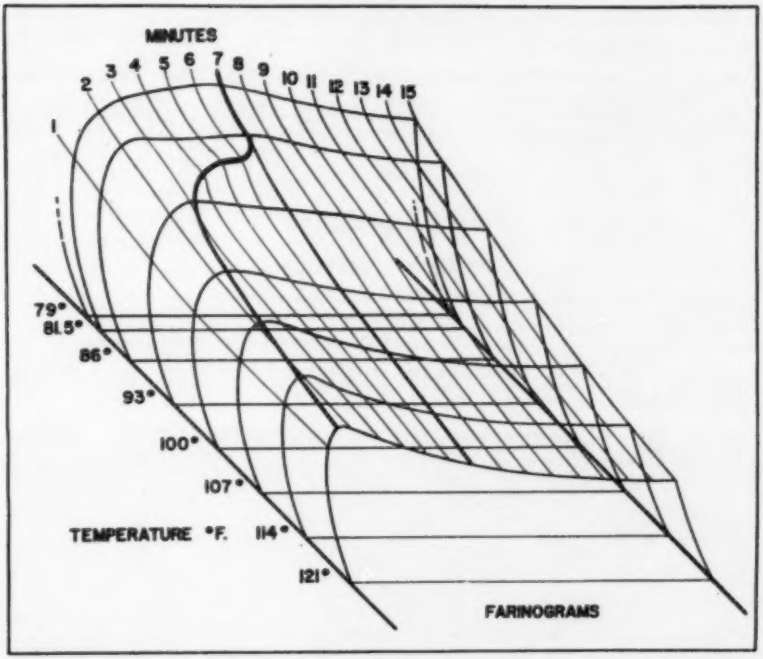

Fig. 4. Three-dimensional interpretation of farinogram curves (constant absorption).

of the dough (5). The new peaks formed to the left at higher temperatures represent other factors involved in the initial mixing of the dough ingredients. These factors are present in all of the doughs but are partially obscured by the farinogram peaks at normal temperatures.

New farinogram interpretations need to be developed for doughs at temperatures other than those previously considered as normal. This is especially important in the area of higher temperature conditioning and processing of doughs used by the more recent developments in continuous bread production.

Acknowledgment

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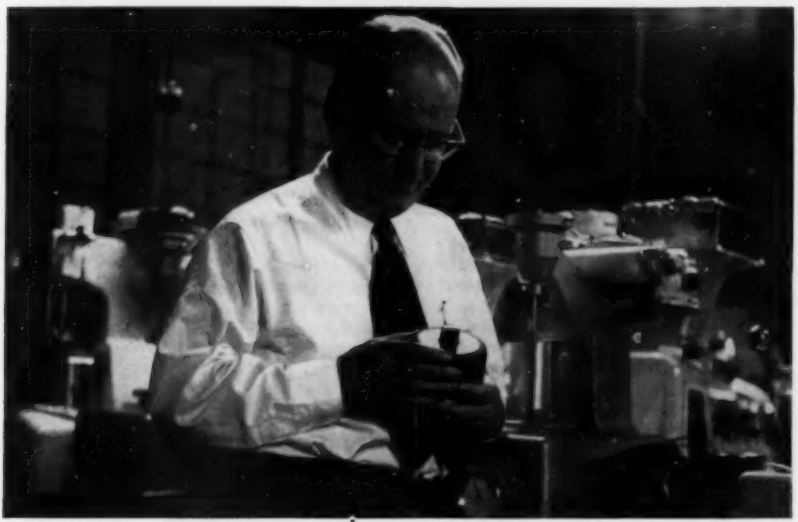

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